The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Chemical Nature of Toxohormone (Nakahara)[1]

First Report

A Globulin-like "Toxohormone" from Cancerous Ascitic Fluid (A.P. I.)*

By

Hajime Masamune, Shigeru Tsuiki, Sigetosi Kamiyama,
(正宗 一) (立木 繁) (加美山 茂利)

Sin-iti Abe, Mituo Haga, Hiroya Kawasaki,
(阿部 辰一) (芳賀 光雄) (川崎 裕也)

Hirosi Kaketa and **Shoichi Abe**
(掛田 博) (阿部 正一)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, January 10, 1958)

Not a few investigators[2)-10)] were and are engaged in establishing the chemical nature of "toxohormone", with conflicting conclusions, some saying it to be a peptide, some to be a nucleic acid, some to be a nucleoprotein, and some to be a low-molecular base. We once proposed[11)] that "toxohormone" is a compound closely resembling uric acid. However, in a Batteli-Stern method, which was applied to assay the liver catalase-depressing activity in that study, the reaction flask was shaken by the hand, giving not necessarily objective results, so that we retract transitorily the opinion until it is confirmed by a reinvestigation. Recently we changed the technique of assay that the flask may be shaken by the aid of a motor at a definite frequency. The experiments since then disclosed unexpected facts, which may be summarized as follows:— 1) There exist a number of toxohormones, strong and weak. 2) Their sources are not restricted to cancerous materials, although the toxohormones from non-cancerous materials are generally weaker in activity and less in yield than those from corresponding cancerous materials. 3) Most of toxohormones contain a hexosamine-containing carbohydrate moiety whether large or small.

The substances, which have been found to act as toxohormones, will be informed of in a series of papers. The present account deals with a globulin-like protein from cancerous ascitic fluids with original seat in stomach. It possesses toxohormone- and KIK-activity simultaneously. Since the liver catalase-depressing activity was distributed among different

* 13th report of Masamune and co-workers' "On Proteins and Amino Acids." 4th report of "Chemical Nature of KIK Factors".

fractions in several trials of isolating " toxohormone ", the presence of more than one toxohormone even in a cancerous ascitic fluid seems highly probable.

Experimental

Methods of Assays

Assay of catalase-depressing activity *in vivo*

Mice of DD strain weighing 10–20 g. were used in most cases, and that male or female animals alone of similar weights for one test-set including two controls below. The substance to be assayed was dissolved or homogenized in water, adding alkali to pH 8 or less when necessary, and 0.5 or 1 cc. of the solution or homogenate was injected per caput intraperitoneally. The blank control was processed by replacing the same volume of plain or alkaline water for the solution or homogenate. The animals were killed by decapitation 24 hours after the injection, and bled well. 0.1 g. of liver or kidney or 0.1 cc. of blood was then homogenized in 10 cc. of 1/15 *M* phosphate buffer of pH 7.0 previously chilled with ice, and 0.3 cc. of the tissue homogenate was subjected to determination of catalase activity as described by Nakahara and Fukuoka.[1] Namely, it was diluted with 0.7 cc. of the same phosphate buffer and let react with 10 cc. of 3% H_2O_2 in a Battelli-Stern apparatus for accuratey 5 minutes, shaking 220 times per minute by means of a motor at room temperature (10–20°C). The volume of oxygen gas evolved was recalculated into that at the standard conditions. In any test-set 3–5 animals were used each for the blank control, for the control with a substance possessing a known liver catalase-depressing activity, and for each dose of the unknown, and the average volumes of oxygen in the main run were converted into percentages of the corresponding volume in the blank control. Three or more such test-sets were carried out to determine the toxohormone activity of a substance. The average percentage thus given divided with 100 will be referred to as " depressed catalase activity ". " Depressed catalase activities " less than 0.75, we assume, signify positive toxohormone reaction.

The volume of oxygen evolved under the above conditions varies proportionally or not proportionally depending on the individuality of animal, with, but always as a linear function of the amount of tissue. Fig. 1*a* shows oxygen gas volumes in an example, in which a mouse liver homogenate prepared as above was diluted 6/5-, 6/4-, 6/3-, 6/2- and 6/1-fold with the phosphate buffer above, and 0.3 cc. portions of the original and diluted homogenates were subjected to the procedure above of catalase activity determination. k_5 (5 signifies the reaction time in minutes) was calculated from equation $k=\frac{1}{t}\log\frac{a}{a-x}$, where a is 2000 cc. (O_2), and x is the volume in cc. of oxygen gas evolved as converted into the normal state. Fig. 1*b* shows that k_{25} (25 signifies the reaction time in seconds) values calculated from the volumes in cc. of 0.02 *N* $KMnO_4$, which were used in titration of mixtures of 5 cc. of 0.01 *M* H_2O_2 in

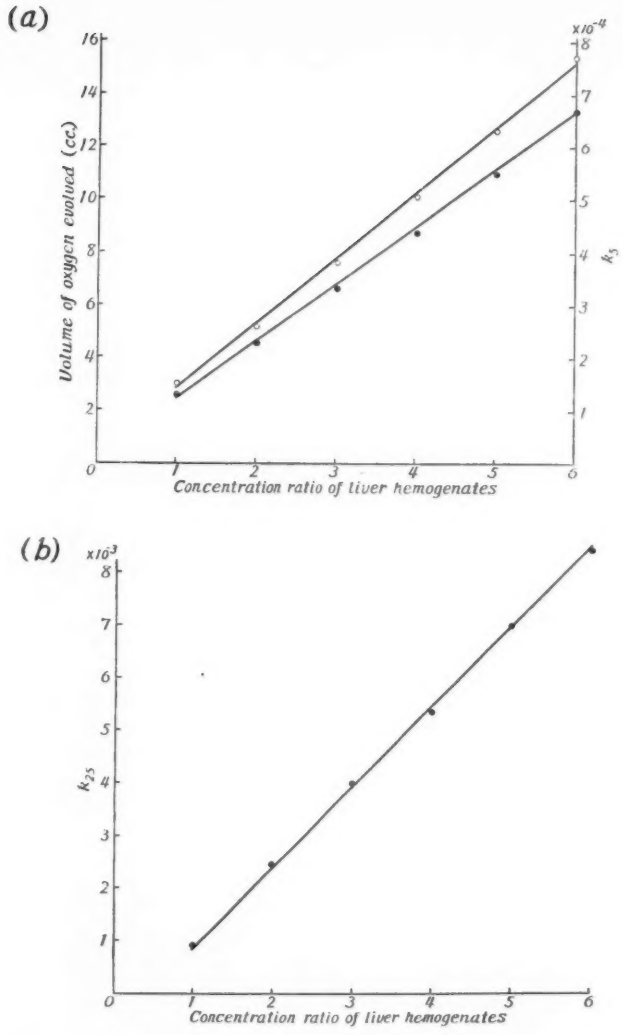


Fig. 1. (*a*) Volumes in cc. of oxygen gas produced from H_2O_2 at 14°C by various amounts of a mouse liver tissue as measured gasometrically by the procedure described and k_5 values calculated therefrom. ○=volume of oxygen gas evolved. ●=k_5 value.

(*b*) k_{25} values calculated from the amounts of H_2O_2 consumed at 0°C by various amounts of the mouse liver tissue same as in (*a*) as determined by the titrimetric method.

0.01 *M* phosphate buffer of pH 6.8 and 0.1 cc. of the above liver homogenates, both chilled to 0°C, after standing for 25 seconds at 0°C and subsequently acidifying with 5 cc. of 10% H_2SO_4. To measure the initial substrate amount, 0.1 cc. of one of the homogenates was added to 5 cc. of the substrate solution, acidified with 5 cc. of 10% H_2SO_4 and titrated similar to above. Cf. Shirakawa.[12] The k_{25} values given practically fall on a straight line. These facts afford an evidence that the relative strengths of catalase activities can be determined quite accurately by the gasometrical procedure above.

Assay of liver-catalase inhibition *in vitro*

0.3 cc. of a tissue homogenate prepared as above with a normal mouse liver was incubated with 1.5 cc. of a solution or homogenate in 1/15 *M* phosphate buffer of pH 7.0 of the unknown for 60 minutes at 20°C, and the catalase activity was measured also as above. Two controls were effected replacing the buffer solution for the solution of the unknown, one with incubation and the other without incubation.

Assay of anemia-inducing potency

This potency was measured as described by Masamune and Kawasaki.[13]

Preparation Procedure of A.P. I

Cancerous ascitic fluids with original seat in stomach were fractioned, taking not only " depressed liver catalase activity " but also yield as indications, because the catalase activity could not be concentrated in one fraction at the second and following stages. An example will be detailed.

First stage. 7 *l.* of the cancerous ascitic fluids united was distilled in vacuo to 1/10 the volume, and 5 volumes of abs. alcohol were added. The deposit was washed with alcohol and dried. 150 g. of a power was given.

Second stage. It was taken up in 400 cc. of water, adjusted to pH 7.0 with sodium hydroxide, and after addition of 1.6 *l.* of abs. alcohol, boiled under reflux for 2 hours. The insoluble part was centrifuged off while hot, and extracted twice more in a similar manner. The solutions were distilled together in vacuo to a syrup, exhausted with ether and precipitated with excessive alcohol, followed by drying. Yield 5.2 g.

Third stage. 4 g. of the fraction was kneaded with 200 cc. of water, and dissolved by slight alkalinization with sodium hydroxide, and the pH value of the solution was lowered with hydrochloric acid to 2.0, whereby the deposit occurring intermediately dissolved again. The solution was then electrodialyzed in a Pauli apparatus. A precipitate began soon to occur. After 4 days, it was separated, washed with alcohol and dried. 2.5 g. was the yield here.

Fourth stage. 2 g. of it was dissolved in 30 cc. of water and alkalinized to pH 9.0 with sodium hydroxide. The small insoluble part was rejected after centrifugation. The supernatant was neutralized with hydrochloric acid, and after removing a tiny deposit, fractionally precipitated by addition of abs. alcohol in such portions as to raise the alcohol concentration as much as 10% at a time.

Most of the substance precipitated at the concentration range of alcohol of 30–40%. It was collected, washed with alcohol and dried. Yield 1.67 g.

Fifth stage. 1.2 g. of the substance given was dissolved in 100 cc. of water, and solid ammonium sulfate was added in portions increasing the molarity of ammonium sulfate 0.1 by 0.1. All the successive precipitates were separately dissolved in water, dialyzed, condensed and precipitated again with alcohol, followed by drying. The largest fractions were given when the ammonium sulfate concentration was increased to 1.3 *M* and to 1.4 *M*. These were combined, because they gave single boundaries with identical mobilities on electrophoresis and showed similar toxohormone activities, though not strong. Yield 0.66 g.

Properties of the Final Product

The substance was dried to a constant weight in vacuo over $CaCl_2$.

1) It proved homogeneous electrophoretically as well as ultracentrifugally.[14] Electrophoretic examination was carried out in a Tiselius apparatus (Type HTD-1, Hitachi) and the ultracentrifugal in a Spinco ultracentrifuge Model E. See Figs. 2 and 3.

2) Toxohormone activity. As shown in Table I, 5 mg. of the substance undoubtedly depresses the liver catalase activity *in vivo*. Table II and III show that the substance does not lessen the kidney- and blood-catalase activities *in vivo* nor liver catalase activity *in vitro*.

3) Anemia-inducing potency. 0.5 mg. of the substance caused a little more than 10% decrease of the number of erythrocytes (Table IV).

4) Test-tube tests. Biuret #, Molisch +, Elson-Morgan +. Phosphorus was not detected.

5) Paper partition chromatography of sugars and amino acids. a) Sugars. 10 mg. of the substance was heated with 1 cc. of 1 *N* H_2SO_4 in a sealed tube at 100°C for 5 hours. The hydrolysate was neutralized to pH 4.6 with 3.4 cc. of the satd. baryta. The precipitate was centrifuged off and washed with three

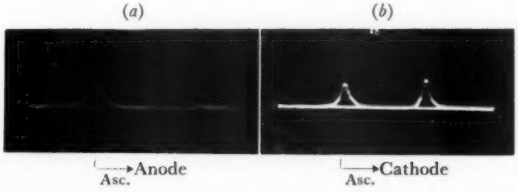


Fig. 2. Electrophoresis patterns of 1% solutions of A.P. I in veronal buffer, pH 8.6, I=0.2 (*a*) and glycine buffer, pH 2.0, I=0.2 (*b*); current 5 mA; temperature 10°C. Exposure 120 min. after starting current. Mobility:

(a) $\dfrac{(-1.30)\times 0.01062\times 0.18}{7200\times 0.005}=-6.90\times 10^{-5}$ cm²/volt/sec

(b) $\dfrac{(+1.14)\times 0.01528\times 0.18}{7200\times 0.005}=+8.71\times 10^{-5}$ cm²/volt/sec

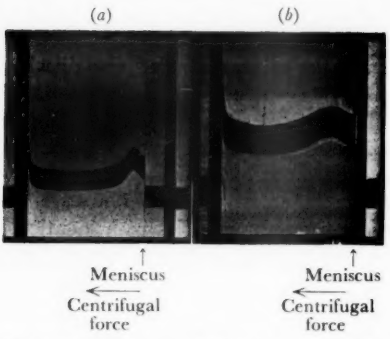


Fig. 3. Sedimentation diagrams of 1% solutions of A.P. I. (*a*) Veronal buffer, pH 8.6, I=0.2; 42 min. after reaching full speed 57,600 rev./min.; rotor temperature 8.6°C; bar angle 70°. (*b*) Glycine buffer, pH 2.0, I=0.2; 44 min. after reaching full speed 59,500 rev./min.; rotor temperature 11.2°C; bar angle 45°.

2–3 cc. portions of warm water (70°C), and the supernatant and washings were evaporated in a vacuum desiccator containing $CaCl_2$ to about 1 cc., whereby again a precipitate appeared. This deposit was filltered off with suction and washed. The filtrate and washings here were evaporated together to dryness in a similar manner. The residue was dissolved in 0.05 cc. of water and applied on a 12×60 cm. sliver of Toyo Roshi filter paper No. 51 to chromatograph with the solvent mixture of butanol, pyridine and water of Masamune and Yosizawa.[15] As shown in Fig. 4, galactose and mannose were detected. No spot of hexosamine was indicated, although the substance before hydrolysis gave the positive Elson-Morgan reaction. It is probably ascribed to the abundant amount of co-present amino acids.

b) Amino acids. 5 mg. of the substance was hydrolyzed with 1 cc. of 6 *N* HCl by heating in a sealed tube at 100°C for 24 hours. The hydrolysate was

TABLE I

Liver-catalase Depression *in vivo* by A.P. I

The average volume of O_2 evolved for each dose in each test-set is expressed in per cent of the corresponding volume in the blank control.

No. of test-set	Amount of substance injected (mg.)		
	5	3	1
1	57.7	83.6	91.3
2	59.3	69.5	93.1
3	65.2	69·5	89.2
Depressed catalase activity	0.61	0.74	0.91

TABLE II

Effect *in vivo* of A.P. I on Kidney- and Blood-catalase

5 mg. amounts of the substance were injected into mice. Liver catalase activities were also determined for comparison.

No. of mouse	Kidney	Blood	Liver
Oxygen evolved (cc.) in blank experiments			
101	2.8	1.4	11.1
102	3.0	1.0	8.2
103	3.5	2.2	10.9
Average	3.1	1.53	10.07
Oxygen evolved (cc.) in main experiments			
104	2.3	1.1	4.9
105	3.0	1.9	6.8
106	3.7	2.2	8.0
Average	3.0	1.73	6.57
(% of the control value)	(97)	(112)	(65)

TABLE III

Effect *in vitro* of A.P. I on Liver Catalase

The liver homogenate was incubated with 5 mg. of the substance.

No. of mouse	Liver catalase activity (oxygen evolved in cc.)		
	Control I (No incubation)	Control II (Incubated without the substance)	Main (Incubated with the substance)
107	12.2	10.0	10.1
108	13.0	9.0	9.8
109	15.6	10.2	11.7
Average	13.6	9.7	10.6 (109% of Control II value)

freed from most of the hydrochloric acid by repeated evaporations in vacuo over solid caustic soda, adding water from time to time, as used to in this laboratory. The final dried residue was taken up in 0.1 cc. of water. And 0.02 cc. of the solution was sampled on a 30×30 cm. square of Toyo Roshi filter paper No. 50, and chromatographed in a usual manner. The chromatogram showed spots at the positions corresponding to aspartic and glutamic acids, serine, glycine, threonine, alanine, tyrosine, phenylalanine, leucine and/or isoleucine, proline, lysine, histidine and arginine and a spot of a probable peptide. See Fig. 5.

5) Quantitative analyses. N (micro Kjeldahl) 13.5%, hexosamine (Masamune and Yosizawa[16]) 1.5%, mannose (Masamune and Sakamoto[17]) 1.3%,

TABLE IV

Anemia-inducing Potency of A.P. I

Assay No.	Rabbit			Amount of the substance injected* per kg. body weight (mg.)	Number of red cells						
	No.	Body wt. (kg.)	Sex		Before inj.	Time after inj. (hrs.)					Maximum decrease (%)
						1	2	3	4	5	
1	1	2.5	♂	1.0	661	624	621	**547**	576	576	17
2	2	2.3	♂	„	689	711	653	581	**488**	530	28
3	3	2.2	♂	0.5	569	571	555	526	**486**	501	14
4	4	2.4	♀	„	726	806	794	**629**	651	665	13
5	5	3.0	♀	0.1	554	548	538	536	**523**	541	6
6	6	2.5	♀	„	547	525	**517**	535	530	521	6

* Intravenously.

Fig. 4

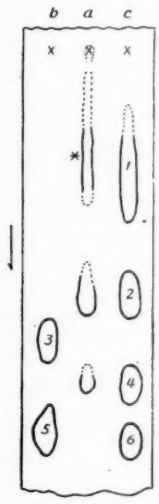


Fig. 5

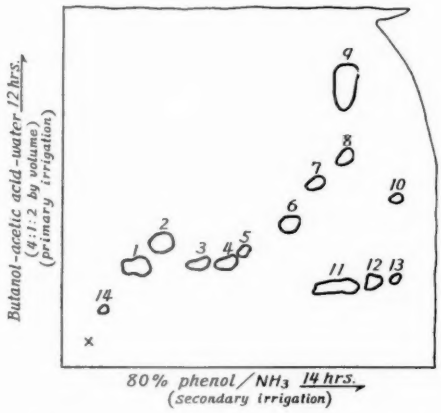


Fig. 4. Sugar paper partition chromatogram of A.P. I ; Irrigation solvent butanol-pyridine-water (5:3:2 by volume), temperature 21°±1°C ; duration 24 hrs. Spray reagent 2% aniline phthalate in moist butanol. Reference runs effected with a 0.01 cc. portion of 1% solutions with respect to individual sugars.

a : the hydrolysate of A.P. I. *b*, *c* : reference run. *1* galactosamine and glucosamine, *2* galactose, *3* glucose, *4* mannose, *5* xylose, *6* L-fucose, * yellowish.

Fig. 5. Amino acid paper partition chromatogram of A.P. I. Temperature of irrigation 21°±1°C. Spray reagent 0.2% ninhydrin in moist butanol.

1 aspartic acid, *2* glutamic acid, *3* serine, *4* glycine, *5* threonine, *6* alanine, *7* tyrosine, *8* phenylalanine, *9* leucine and/or isoleucine, *10* proline, *11* lysine, *12* histidine, *13* arginine, *14* a probable peptide.

galactose (Masamune and Sakamoto[17]) 0.6%, pentose (Masamune and Sakamoto[17]) 0%, methylpentose (modified Dische and Shettles method[18]) 0%, ash 0%. Pentose and methylpentose were spectrophotometrically determined to ascertain the negative result of paper partition chromatography which was not well reliable because of the tiny amount of the polysaccharide in the protein.

6) Optical rotation in 0.005 *N* NaOH (pH of the solution 8.0)

$$[\alpha]_D^{t} = \frac{-0.7375 \times 100}{1.007 \times 1} = -73.2°$$

7) Spectral absorption. A Beckman type spectrophotometer (Type EPU-2A Hitachi) was used. The substance weakly absorbed ultraviolet light with a flat peak extending over 275–280 mμ due to cyclic amino acids, but without any peak at 260 mμ. See Fig. 6.

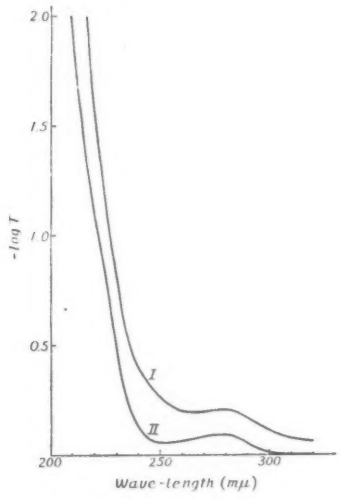


Fig. 6. Spectral absorption of A.P. I. 0.1 cc. of the solution used for measurement of optical rotation was made up to 10 cc. with water (pH 7.2) (I) or with 0.005 *N* NaOH (pH 8.0) (II).

8) Specific refraction. The solution in 6) was examined in an Abbe refractometer at 17.5°C.

n 1.33513, n_o 1.33324, $d^{17.5}/_4 = 1.0035$, $d_o^{17.5}/_4 = 1.0003$, $p = 1.007$

$$[n]_D^{17.5} = \frac{1.33513^2-1}{1.33513^2+2} \times \frac{1}{1.0035} \times \frac{100}{1.007} - \frac{1.33324^2-1}{1.33324^2+2} \times \frac{1}{1.0003} \times \frac{98.993}{1.007} = 0.272$$

9) It is group inactive within ABO classification.

Summary

1. A " toxohormone " possessing anemia-inducing potency has been

isolated from cancerous ascitic fluids with original seat in stomach in an electrophoretically as well as ultracentrifugally homogeneous state, and its properties, physical and chemical, examined.

2. Our findings on " toxohormone " are briefly sammarized.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References and Notes

1) Nakahara & Fukuoka, Gann, 1949, **40**, 45.
2) Greenfield & Meister, J. Nat. Cancer Inst., 1951, **11**, 997.
3) Fukuoka & Nakahara, Gann (Jap.), 1953, **44**, 71.
4) Nakahara & Fukuoka, *ibid.*, 1950, **41**, 47.
5) Nakahara & Fukuoka, *ibid.*, 1954, **45**, 67.
6) Sato, Tohoku Igaku Zassi (Jap.), 1957, **56**, 578.
7) Nakagawa, Kosuge & Tokunaka, Gann, 1955, **46**, 585.
8) Ono, Sugimura & Umeda, *ibid.*, 1955, **46**, 617.
9) Ono, Umeda & Sugimura, *ibid.*, 1957, **48**, 91.
10) Kuzin, Sharoukhova & Chudinova, Biokhimiya, 1955, **20**, 126 ; Cited in C. A., 1955, **49**, 9135.
11) Masamune, Yosizawa & Kaketa, Gann, 1956, **47**, 300.
12) Shirakawa, Nippon Nogeikagaku Kaishi (Journal of the Agricultural Chemical Society of Japan) (Jap.), 1951, **24**, 125.
13) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 372.
14) The writers are indebted to Mr. Y. Amano in the Bacteriological Department for the ultracentrifugal examination.
15) Masamune & Yosizawa, Tohoku J. Exp. Med., 1953, **59**, 1.
16) Masamune & Yosizawa, *ibid.*, 1957, **65**, 169.
17) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
18) Masamune & Kawasaki, *ibid.*, 1956, **63**, 381–382.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Biochemical Studies on Carbohydrates

CCXI. A KIK Factor (V) from Cancerous Urine and the Corresponding Substance from Normal Urine*

By

Hirosi Kaketa

(掛田　博)

From the Medico-chemical Institute, Tohoku University, Sendai; Director: Prof. H. Masamune

(Received for publication, January 10, 1958)

Urines of various cancer patients were fractioned, taking KIK reaction as an indication. The electrophoretically homogeneous products were found identical with one another, being all a glucidamin regardless of the site of tumour. It differs from the corresponding fraction of normal urine, which proved to be devoid of KIK activity, in quantitative composition, optical rotation, ultraviolet absorptivity and electrophoretic mobility.

Experimental

Fractionation of Urines, Cancerous and Normal

Urines were collected from stomach-, liver-, lung- and mamma-cancer patients and normals separately, and their mucoprotein fractions were prepared by a modification of Nakagawa and Kasai procedure[1] as follows:—

2–5 *l.* of urine was made slightly acidic (pH 6.0) with 40% acetic acid or 10% NaOH and filtered, and the filtrate was added to with 60 cc. of 25% benzoic acid in 10% NaOH per liter and adjusted to pH 9.0. The white viscous precipitate was rejected after centrifugation, and the yellowish-brown, transparent supernatant was acidified to pH 4.0 with 25% HCl, whereby an abundant grayish precipitate appeared. After standing for 1–2 hours, the deposit was brought onto a suction funnel, stirred in 5 volumes of acetone, washed with abs. alcohol twice and with ether once and dried. A brown powder (Kaketa fraction) was given—*Fr. a.* Average yield per liter of urine amounted to 0.16 g. in the cases of cancerous urines and to 0.08 g. in the case of normal urine.

5 g. of *Frs. a* was taken up in 125 cc. of water, and 10% NaOH was added to pH 9.0. The centrifuged supernatant (dark brown) and washing were acidified to pH 4.5, and the brown precipitate set aside. The mother fluid was neutralized to pH 6.0–7.0, 1/2 the volume of 10% trichloroacetic acid was added, and the mixture was stood in an ice-chest for 5–6 hours. The brownish green precipitate was centrifuged off and rejected, and to the yellowish green,

* 5th report of Masamune and co-workers' "Chemical Nature of KIK Factors."

transparent supernatant (pH<1.0) were added 1.5 volumes of acetone. The mixture was placed in the ice-chest for 4–5 hours, and after removing the deposit (brownish-green), distilled in vacuo at below 30°C to about 1/3 the volume to expel most of the acetone. The condensate was dialyzed against running water for 1 or 2 days until it turned neutral. When a deposit was given in the mean time, it was eliminated. The solution was further concentrated in a similar manner to a syrup and precipitated with abs. alcohol containing some anhydrous sodium acetate, followed by standing in the ice-chest for 2–3 hours. Centrifuged. The centrifugate was washed with two changes of abs. alcohol and with ether and dried. 420–800 mg. of a brownish gray powder was given—*Fr. b.*

1 g. of *Frs. b* was dissolved in 100 volumes of water, and the dark brown solution (pH 6.2–6.5) was fractionally precipitated by addition of anhydrous sodium sulfate in portions. The precipitates at the concentration range of sodium sulfate from 0 to 11.1% (w/v) were rejected, and those at the sodium sulfate concentrations till 27.2% were taken. These were dissolved in 100 volumes of water, dialyzed against running water for 1 day, passed through colnmns (0.7×25 cm.) of Amberlite IR 120 (H form) and of Amberlite IR 45 (OH form) in turn and washed with water. The second effluent and washing were distilled in vacuo to syrupy consistency, precipitated with a mixture of equal volumes of abs. alcohol and of ether, washed with ether and dried. 280–510 mg. of a grayish powder was given—*Fr. c.*

Next, it was dissolved in such a volume of 2% $Ba(AcO)_2$ as gives a 1.5% solution, and chilled on ice. Abs. alcohol was added in portions to the solution continuously cooled, and the precipitates at the alcohol concentration range from 10 to 25% were taken and dried. These were reprecipitated together. This fraction was freed from parts of the neighbouring fractions which had been carried down together, by repetitions of the fractional precipitation, and after taking up in about 100 volumes of water, passed through columns of Amberlite IR 120 and IR 45 as above. The second effluent (pH 5.0, yellowish, almost transparent) and washing here were also distilled in vacuo to a syrup, precipitated with a mixture of abs. alcohol and ether, washed with ether and dried.—12–25 mg. of a grayish powder was given. This proved electrophoretically homogeneous as will be illustrated below. The products prepared from cancerous urines were anemia-inducing, and that from normal urine non-anemia-inducing. The average yields from urines of stomach-, liver-, lung- and mamma-cancer patients and normals were 4.8, 6.0, 5.3, 6.1 and 1.2 mg. respectively per 10 *l*. The preparations from the urines of those patients in the order arranged and normals will be referred to as Prep. I, II, III, IV and V. It is noteworthy that the yield from normal urine was remarkably less than those from the cancerous urines.

Properties of the Preparations

They were dried to a constant weight in vacuo over $CaCl_2$.

Electrophoresis. It was examined in a Tiselius apparatus (Type HTD-1 Hitachi). All the preparations showed a single boundary in alkaline and an acid buffer after electrophoresis. See Fig. 1. A mixture of equal amounts of the KIK-active products showed also a single boundary, but that of equal amounts of

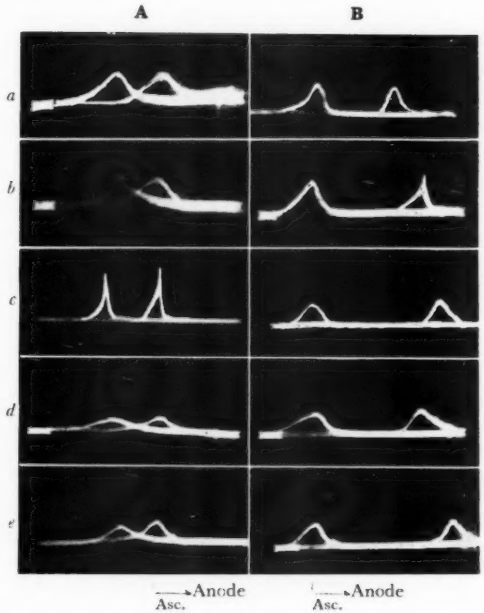


Fig. 1. Electrophoresis patterns of 1% solutions of Prep. I (*a*), II (*b*), III (*c*), IV (*d*) and V (*e*) in phosphate buffer pH 7.8, I=0.2 (**A**) and acetate buffer, pH 4.6, I=0.2 (**B**) ; current 4 mA ; temperature 20°C. Exposure 120 min. in **A** and 60 min. in **B**. Mobility :

Prep. I at pH 7.8 $\frac{(-1.92)\times 0.0032\times 0.18}{7200\times 0.004}=-11.1\times 10^{-5}$ cm²/volt/sec

at pH 4.6 $\frac{(-0.4)\times 0.0067\times 0.18}{3600\times 0.004}=-3.4\times 10^{-5}$ "

Prep. II at pH 7.8 $\frac{(-1.92)\times 0.0032\times 0.18}{7200\times 0.004}=-11.1\times 10^{-5}$ "

at pH 4.6 $\frac{(-0.36)\times 0.0067\times 0.18}{3600\times 0.004}=-3.0\times 10^{-5}$ "

Prep. III at pH 7.8 $\frac{(-1.92)\times 0.0032\times 0.18}{7200\times 0.004}=-11.1\times 10^{-5}$ "

at pH 4.6 $\frac{(-0.4)\times 0.0067\times 0.18}{3600\times 0.004}=-3.4\times 10^{-5}$ "

Prep. IV at pH 7.8 $\frac{(-1.92)\times 0.0032\times 0.18}{7200\times 0.004}=-11.1\times 10^{-5}$ "

at pH 4.6 $\frac{(-0.36)\times 0.0067\times 0.18}{3600\times 0.004}=-3.0\times 10^{-5}$ "

Prep. V at pH 7.8 $\frac{(-1.68)\times 0.0032\times 0.18}{7200\times 0.004}=-9.6\times 10^{-5}$ "

at pH 4.6 $\frac{(-0.56)\times 0.0067\times 0.18}{3600\times 0.004}=-4.7\times 10^{-5}$ "

the product from urine of stomach-cancer patients and that from normal urine two boundaries.

Anemia-inducing potency. It was measured as described by Masamune and Kawasaki[2], that is, various amounts of the preparations were dissolved in 1 cc. of physiological saline and injected intravenously into rabbits, and the number of erythrocytes was calculated before and at intervals during 5 hours after the injection. Table I illustrates details of the result. The KIK factor preparations decreased the number of erythrocytes by more than 10%, when injected in a dosis of 0.1 mg. per kg. body weight. In the case of the preparation from the urine of stomach-cancer patients, where lesser amounts were examined, even 0.05 mg. per kg. body weight was found to cause 10% decrease of the number of erythrocytes.

TABLE I

Anemia-inducing Potency of the Preparations

Rabbits Nos. 1, 7, 3, 6 and 10 were used at an interval of 3 months.

Assay No.	Rabbit			Material injected	Dosis per kg. body wt. (mg.)	Number of red cells						
	No.	Body wt.(kg.)	Sex			Before inj.	Time after inj. (hrs.) 1	2	3	4	5	Maximum fluctuation (%)
1	1	2.1	♂		0.1	760	740	732	631	**587**	619	−23
2	2	2.1	♂		,,	703	692	680	560	**521**	580	−26
3	3	2.8	♂	Prep. I	0.05	649	630	619	**535**	564	570	−18
4	4	2.0	♂		,,	666	635	613	601	**592**	600	−11
5	5	2.8	♂		0.02	684	680	679	677	626	**620**	− 9
6	6	2.7	♀		,,	678	682	670	670	628	**619**	− 9
7	7	2.2	♀		0.1	768	792	720	680	**673**	700	−12
8	8	2.5	♀	Prep. II	,,	774	772	738	638	626	**608**	−21
9	9	2.5	♀		,,	830	788	760	759	**613**	621	−26
10	10	2.3	♀		,,	759	746	698	672	695	**647**	−15
11	11	2.1	♂		,,	579	579	510	454	**442**	491	−23
12	12	2.6	♀		0.5	602	590	570	**493**	534	550	−19
13	13	2.3	♀	Prep. III	0.25	644	651	590	**505**	541	520	−22
14	14	2.0	♀		0.1	679	609	580	**492**	544	500	−27
15	15	2.2	♂		,,	671	590	543	**511**	525	590	−24
16	16	3.0	♂		,,	762	750	732	625	**598**	610	−22
17	17	1.9	♀		0.1	545	560	521	449	**441**	517	−19
18	18	1.9	♂	Prep. IV	,,	641	713	608	548	574	**533**	−17
19	19	2.1	♂		,,	707	728	596	628	**578**	595	−18
20	1	2.2	♂		,,	698	680	673	**595**	602	611	−15
21	7	2.3	♀		1.0	697	680	703	740	720	**692**	± 0
22	3	3.0	♂	Prep. V	,,	660	668	670	**631**	653	701	− 5
23	6	2.9	♀		,,	604	592	607	618	**601**	603	± 0
24	10	2.4	♀		,,	644	648	620	690	614	**599**	− 7

Solubility. The different preparations proved similar in this respect. They dissolve in water, plain, acidified and alkalized, and 90% phenol, and precipitate from water on saturation with sodium sulfate to 33% (w/v) at 35°C and on

saturation with ammonium sulfate at room temperature. They dissolve also in glacial acetic acid, when previously kneaded into a paste with a small quantity of water, but does not immediately. Their 0.5–1% solutions in water are not precipitated with barium acetate and cupric chloride but are with lead acetate.

Test-tube tests. The results are tabulated as follows.

Test	Preparation				
	I	II	III	IV	V
Molisch	++	++	++	++	++
Biuret	+	+	+	+	+
Osaki-Turumi					
Indirect	+	+	+	+	+
Direct	+	+	+	+	+
Bial-Mejbaum	+	+	+	+	+
Test for P	−	−	−	−	−

Paper partition chromatography. Sugars. 12–14 mg. of substance was sealed with 1.2–1.4 cc. of 1 *N* H_2SO_4 in a tube and heated at 100°C for 5 hours. The hydrolysate was neutralized with the satd. $Ba(OH)_2$ to pH 5.0–6.0 and centrifuged. The centrifugate was washed with three 2 cc. portions of water warmed to 70°C. The supernatant and washings were united and filtered with suction, and the filtrate was evaporated in vacuo over $CaCl_2$ to dryness. The residue was dissolved in 0.08 cc. of water, and the halves were applied on two 12×60 cm. slivers of Toyo Roshi filter paper No. 2 separately to chromatograph one with the butanol-pyridine-water mixture and the other with the *n*-butylacetate-acetic acid-ethanol-water mixture according to Masamune and Yosizawa[3]. In all of the preparations, galactosamine, glucosamine, galactose, mannose and xylose and/or lyxose were detected (Fig. 2). In addition, another pair of chromatograms were developed, starting with a hydrolysate of a larger amount (10.3 mg.) of a mixture of equal amounts of the cancerous products. Here, the *n*-butylacetate-acetic acid-ethanol-water chromatogram showed a very weak spot of L-fucose, but the butanol-pyridine-water chromatogram no spot of glucose. See Fig. 2.

Amino acids. 2 mg. of substance was heated with 0.8 cc. of 6 *N* HCl at 100°C for 24 hours, and the hydrolysate was freed from most of the hydrochloric acid by evaporation in a vacuum desiccator, containing anhydrous calcium chloride and solid caustic soda, at 40°C, adding a few drops of water from time to time. When the solution showed a pH value above 2.6, it was filtered with suction and washed with water. The filtrate and washing were evaporated to dryness and dissolved in 0.06 cc. of water. 0.03 cc. of the solution corresponding to 1 mg. of the substance before hydrolysis was sampled on a 30×30 cm. square of Toyo Roshi filter paper No. 2, and a two-dimensional chromatogram was developed in a usual way and indicated with ninhydrin (Fig. 3). All the preparations including that obtained from normal urine showed spots at the positions corresponding to aspartic and glutamic acids, serine, glycine, alanine, lysine, histidine, arginine, proline, valine and/or methionine, leucine and/or isoleucine and/or phenylalanine, tyrosine, cystine, hexosamine and a peptide.

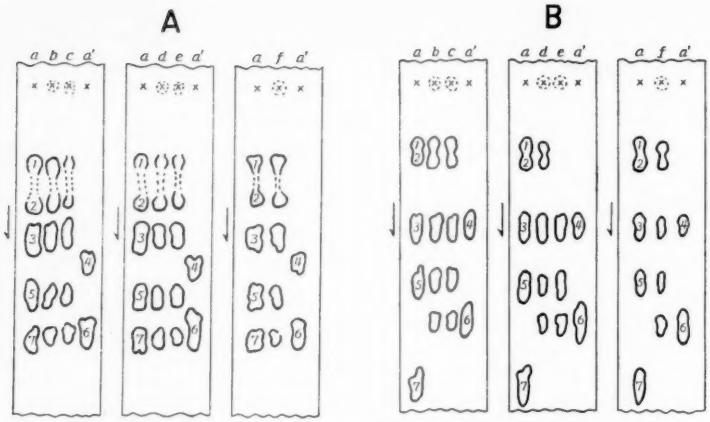


Fig. 2. Sugar paper chromatograms of the hydrolysates of Preps. I–V developed with solvent mixture butanol-pyridine-water (5:3:2 by volume) (**A**) or *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1) (**B**) ; irrigation descending, 25°±1°C ; 24 hrs. Indicator aniline hydrogen phthalate reagent of Partridge. Reference runs effected with 0.02 cc. of 1% solutions with respect to individual sugars.

a, *a'* reference. *b* Prep. I. *c* Prep. II. *d* Prep. III. *e* Prep. IV. *f* Prep. V. *1* galactosamine, *2* glucosamine, *3* galactose, *4* glucose, *5* mannose, *6* xylose, *7* L-fucose.

Quantitative analyses. The analytical figures are embodied in Table II. All the cancerous preparations resemble each other in composition, whereas that from normal urine contains more mannose and less sialic acid.

Optical rotation in water.

Fig. 3A

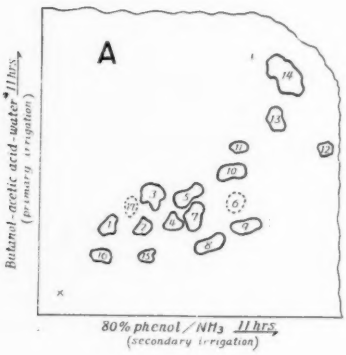
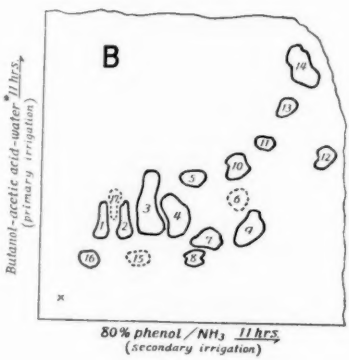


Fig. 3B



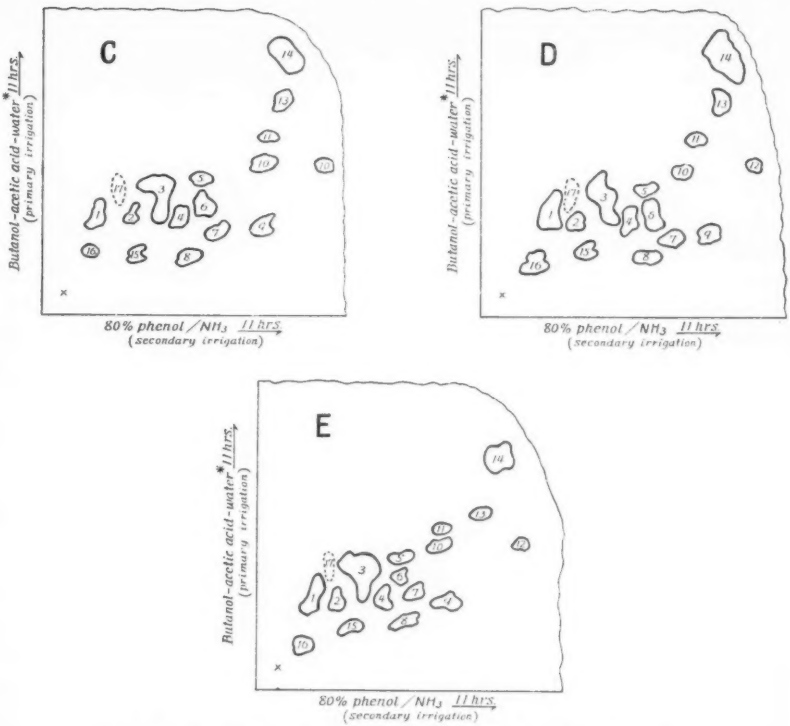


Fig. 3. Amino acid paper chromatograms of the hydrolysates of Prep. I (**A**), II (**B**), III (**C**), IV (**D**) and V (**E**). Temperature of test 22°±1°C. Spray reagent 0.2% ninhydrin in moist butanol. * 4:1:2 by volume.

1 aspartic acid, *2* glutamic acid, *3* serine, *4* glycine, *5* threonine, *6* hexosamine, *7* histidine, *8* lysine, *9* arginine, *10* alanine, *11* tyrosine, *12* proline, *13* valine and/or methionine, *14* leucine and/or isoleucine and/or phenylalanine, *15* cystine, *16* a probable peptide, *17* aspartic acid (a part).

Prep. I: $[\alpha]_D^{17} = \frac{-0.155 \times 100}{0.5 \times 0.25} = -124°$ (pH 5.0)

Prep. II: $[\alpha]_D^{17} = \frac{-0.1525 \times 100}{0.5 \times 0.25} = -122°$ (pH 5.0)

Prep. III: $[\alpha]_D^{17} = \frac{-0.153 \times 100}{0.5 \times 0.25} = -123°$ (pH 5.0)

Prep. IV: $[\alpha]_D^{17} = \frac{-0.1545 \times 100}{0.5 \times 0.25} = -123°$ (pH 5.0)

Prep. V: $[\alpha]_D^{17} = \frac{-0.1255 \times 100}{0.5 \times 0.25} = -100°$ (pH 5.4)

The preparations obtained from various cancerous urines have identical

TABLE II

Composition of the Preparations

Analysis	In per cent					In equivalents per equivalent weight				
	Prep. I	II	III	IV	V	Prep. I	II	III	IV	V
N*	11.7	11.6	11.6	11.9	10.8	25.24	25.30	24.62	25.00	23.75
Hexosamine†	5.9	6.2	5.9	6.1	5.9	1.0	1.0	1.0	1.0	1.0
Galactose‡	4.5	4.7	4.3	4.8	4.6	0.76	0.75	0.71	0.76	0.78
Mannose‡	3.6	3.8	3.2	3.5	4.8	0.58	0.61	0.52	0.57	0.81
L-Fucose§	0.97	1.00	0.92	0.95	1.02	0.17	0.18	0.16	0.17	0.19
Xylose and/or lyxose as xylose//	0.38	0.38	0.35	0.37	0.42	0.07	0.07	0.07	0.07	0.09
Sialic acid¶	7.1	7.0	6.9	7.5	4.4	0.67	0.66	0.65	0.70	0.43
Ash**	2.9	1.6	4.5	3.8	2.2					

* Micro Kjeldahl. † Masamune and Yosizawa.[5] ‡ Masamune and Sakamoto.[6] § Masamune and Sakamoto.[7] // Masamune and Sakamoto.[8] ¶ Werner and Odin (direct Ehrlich)[9]; Calcd. as $C_{11}H_{19}NO_9$. ** Pregl method without use of H_2SO_4.

rotations but that from normal urine a different rotation.

Spectral absorption. 20 mg% solutions in water of the preparations from urines of stomach- and lung-cancer patients and normal urine were examined regarding the ultraviolet absorptivity in a Beckman type spectrophotometer Hitachi (Type EPU-2). The two cancerous preparations showed the same ultraviolet spectrum but the normal absorbed the ultraviolet light less strongly, the maximum of the absorption being at 275 mμ for any of those substances. See Fig. 4.

Discussion

The results with respect to electrophoresis, optical rotation, ultraviolet absorptivity and composition all point to the identity of the cancerous substances here regardless of the site of tumour. These substances differ remarkably from the undialyzable anemia-inducing substances of Sugimoto[1] (KIK factor III) from cancerous ascitic fluid with original seat in stomach and that with original seat in liver, which are the same on their part, as well as from the undialyzable KIK factors (I and II) of Masamune and Kawasaki[2] from gastric juice of stomach cancer patients. We are trying but have not as yet isolated the undialyzable KIK factor or factors from stomach cancer tissue in pure state. Nevertheless, as thus compared, KIK factors from secretions (Cancerous ascitic fluid is assumed to be a partial secretion) and excretions are chemically characterized according to the source but not to the site of original cancer, by which KIK factors of

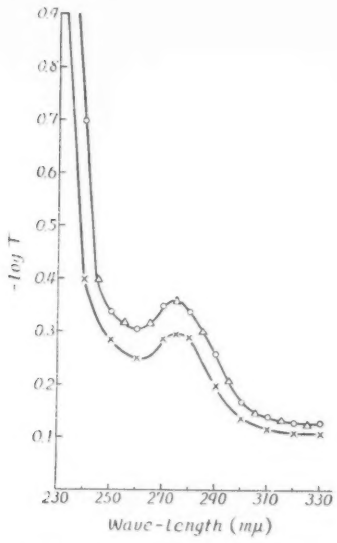


Fig. 4. Spectral absorption of 20 mg% solutions (pH 5.0) of Prep. I (-○-○-), III (-△-△-) and V (-×-×-).

different cancer tissues may or may not be discriminated from one another. The fact strongly suggests that KIK factors in secretions and excretions are formed by the cells normally secreting the corresponding non-anemia-inducing substances which differ from source to source, and the disorder of those cells might be ascribed to an unknown circulating principle or principles elaborated in the tumour.

Summary

1. Urines of stomach-, liver-, lung- and mamma-cancer patients and normals were fractioned, separating an anemia-inducing product from each of the cancerous urines and a non-anemia-inducing product from normal urine in an electrophoretically homogeneous state.

2. The products were investigated chemically and physically. They are all mucoproteins composed of galactosamine, glucosamine, galactose, mannose, L-fucose, xylose and/or lyxose, sialic acid and many amino acids.

3. Those from the various cancerous urines resemble one another in electrophoretic mobility, optical rotation, ultraviolet absorptivity, composition and anemia-inducing potency, in other words, they are identical with one another. That from normal urine differs from those above in all

the respects as well as in yield.

4. The cause of difference among KIK factors according to secretion or excretion is discussed.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References

1) Nakagawa & Kasai, Nippon Naikagakkai Zasshi (Jap.), 1951, **40**, 312.
2) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369.
3) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
4) Sugimoto, *ibid.*, 1956, **64**, 271.
5) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.
6) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
7) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
8) Masamune & Sakamoto, *ibid.*, 1956, **63**, 357.
9) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 270.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Chemical Nature of Toxohormone (Nakahara)*

Second Report

The KIK Factor (V) from Cancerous Urine and the Corresponding Component of Normal Urine Act as "Toxohormones"

By

Hajime Masamune, Hirosi Kaketa and **Sin-iti Abe**

(正宗　一)　(掛田　博)　(阿部辰一)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, January 10, 1958)

This KIK factor was isolated from united urines of stomach-, liver-, lung- and mamma-cancer patients (49, 17, 8 and 18 *l.*, respectively) by the procedure of Kaketa[1]. Yielded was 55 mg. It was examined electrophoretically in a Tiselius apparatus (Type HTD-1, Hitachi) and ultracentrifugally[2] in a Spinco ultracentrifuge Model E, proving homogeneous or essentially homogeneous as shown in Fig. 1 and 2. N (micro Kjeldahl) 11.9%; hexosamine (Masamune and Yosizawa[3]) 5.9%; ash (Pregl method without use of H_2SO_4) 3.1%. The anemia-inducing potency as measured according to Masamune and Kawasaki[4] is detailed in Table I.

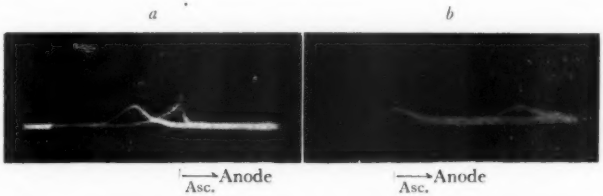


Fig. 1. Electrophoresis patterns of 1% solutions of the preparation from the united cancerous urines; current 4 mA; temperature 20°C. (*a*) Phosphate buffer, pH 7.8, I=0.2; exposure 120 min. after starting current. (*b*) Acetate buffer pH 4.6, I=0.2; exposure 60 min. after starting current. Mobility:

(*a*) $\dfrac{(-1.92)\times 0.0092\times 0.18}{7200\times 0.004} = -11.1\times 10^{-5}$ cm²/volt/sec

(*b*) $\dfrac{(-0.4)\times 0.0067\times 0.18}{3600\times 0.004} = -3.4\times 10^{-5}$ "

As the component of normal urine corresponding to that KIK factor, the balance of the preparation informed of in the Kaketa's article[1] was ap-

* 212th report of Masamune and co-workers' "Biochemical Studies on Carbohydrates".

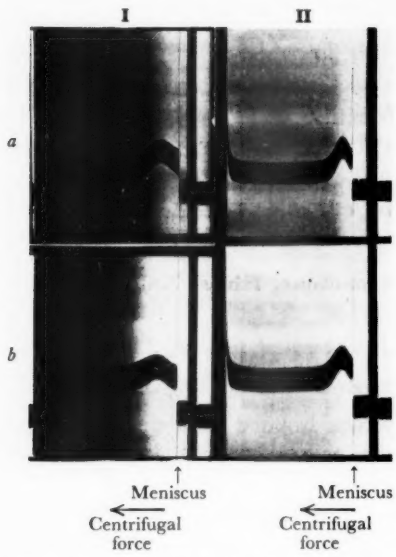


Fig. 2. Sedimentation diagrams of 1% solutions of the preparation from the cancerous urines (**I**) and that from normal urine (**II**) in phosphate buffer, 7.8, I=0.2 (*a*) and acetate buffer, pH 4.6, I=0.2 (*b*). (**I***a*) 110 min. after reaching full speed 57,792 rev./min.; rotor temperature 13°C; bar angle 65°. (**I***b*) 110 min. after reaching full speed 57,798 rev./min.; rotor temperature 13°C; bar angle 55°. (**II***a*) 60 min. after reaching full speed 59,946 rev./min.; rotor temperature 9.6°C; bar angle 70°. (**II***b*) 64 min. after reaching full speed 59,800 rev./min.; rotor temperature 9.8°C; bar angle 64°.

TABLE I

Anemia-inducing Potency of the KIK Factor Preparation (I)

Assay No.	Rabbit			Dosis per kg. body wt. (mg.)	Number of red cells						Maximum decrease(%)
	No.	Body wt. (kg.)	Sex		Before injection	Time after injection (hrs.)					
						1	2	3	4	5	
1	1	2.0	♂	0.1	672	619	630	**529**	544	591	21
2	2	2.5	♂	"	629	630	601	**506**	629	643	20
3	3	2.8	♂	0.05	573	630	577	532	**489**	502	15
4	4	1.9	♂	"	684	665	623	**593**	668	680	13

plied. Fig. 2 shows also its sedimentation diagrams. To further scrutinize its homogeneity, 10 mg. of the preparation was dissolved in 0.5 cc. of the borax-HCl buffer of pH 8.4 (0.05 *M* sodium tetraborate: 0.1 *N* HCl= 6:4 by volume), added to a 0.9×26 cm. (20 cc.) column of Dowex 1 of

mesh 50–200 equilibrated with the buffer above and developed by the aid of the same buffer at a flow rate of 1 drop per 35 seconds, collecting the effluent in fractions of 15 drops (about 1.5 cc.). The fractions were analyzed by the quantitative biuret reaction of Gornall *et al.*[5] on 1 cc. of them, using the buffer solution for the blank, as well as by quantitative Molisch reaction which was carried out as follows:— A mixture of 0.5 cc. of the unknown with 0.1 cc. of 5% α-naphthol in alcohol is added to with

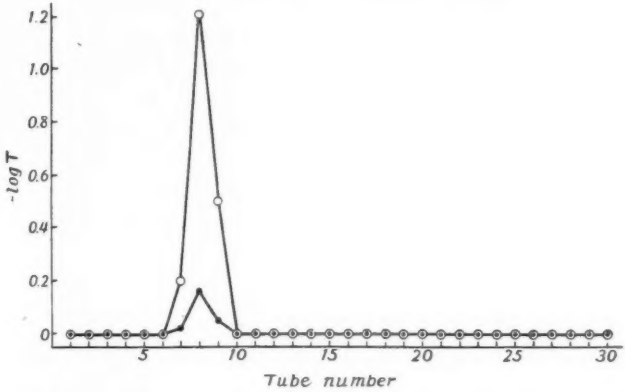


Fig. 3. Column chromatogram of 10 mg. of the fraction of normal human urine corresponding to the urinary KIK factor developed with Dowex 1 and the borax-HCl buffer, pH 8.4. ○ Molisch reaction. ● Biuret reaction.

Table II

Liver Catalase Depression *in vivo* by Preparations, Anemia-inducing (I) and Non-anemia-inducing (II)

The average volume of O_2 evolved for each dose in each test-set is expressed in per cent of the corresponding volume in the blank control.

No. of test-set	Amount of substance injected (mg.)							
	Prep. I (anemia-inducing)				Prep. II (non-anemia-inducing)			
	1	0.5	0.3	0.1	1	0.5	0.3	0.1
1	58.2	66.8	64.8	94.4				
4					65.5	64.7	79.0	93.2
2		66.7	68.0	92.6				
5						71.7	82.3	84.1
3		52.3	85.7	87.7				
6						64.8	78.5	
Depressed catalase activity		0.62	0.73	0.92		0.67	0.80	0.89

TABLE III

Effect *in vivo* on Kidney- and Blood-catalase of Preparations, Anemia-inducing (I) and Non-anemia-inducing (II)

0.5 mg. amounts of Prep. I and of Prep. II were injected into mice. Liver catalase activities were also determined for comparison.

No. of mouse	Prep. I			Prep. II		
	Kidney	Blood	Liver	Kidney	Blood	Liver
Oxygen evolved (cc.) in blank experiments						
101	4.1	2.0	17.0			
107				4.6	2.3	9.5
102	3.4	1.4	17.7			
108				4.4	2.2	12.9
103	2.6	1.0	18.2			
109				3.3	0.8	16.0
Average	3.4	1.5	18.0	4.1	1.8	12.8
Oxygen evolved (cc.) in main experiments						
104	3.8	2.0	8.9			
110				4.5	2.7	6.9
105	3.2	1.3	9.8			
111				4.4	1.6	9.1
106	2.6	1.2	9.6			
112				3.2	1.4	9.2
Average (% of the control value)	3.2 (94)	1.5 (100)	9.6 (53)	4.0 (98)	1.8 (100)	8.4 (64)

TABLE IV

Effect *in vitro* on Liver Catalase of Preparations, Anemia-inducing (I) and Non-anemia-inducing (II)

The liver homogenates were incubated with 0.5 mg. of Prep. I or II.

No. of mouse	Liver catalase activity (oxygen evolved in cc.)					
	Prep. I			Prep. II		
	Control I (No incubation)	Control II (Incubated without the substance)	Main (Incubated with the substance)	Control I	Control II	Main
113	16.3	14.3	16.2			
116				7.6	4.5	6.0
114	17.8	15.5	17.3			
117				11.7	6.0	8.2
115	16.7	13.0	13.9			
118				11.4	3.5	4.5
Average	16.9	14.3	15.8 (111% of Control II value)	8.9	4.7	6.2 (133% of Control II value)

3.5 cc. of conc. H_2SO_4 under cooling on ice, heated in boiling water for 5 minutes, and quickly cooled in iced water, followed by measurement of the extinction at the wave-length of 565 mμ. As shown in Fig. 3, the whole substance appeared forming a peak between the 6th and 10th fractions.

Toxohormone activity. It was revealed that the two substances, anemia-inducing and non-anemia-inducing, are both toxohormone-active, namely, they depress liver catalase (Table II) but not blood- and kidney-catalase *in vivo* (Table III), whereas they do not inhibit or do rather activate or portect from destruction the liver catalase *in vitro* (Table IV). The assays were processed as described by Masamune *et al.*[6]

To add, the substances were found to lack blood group activity within ABO classification.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References

1) Kaketa, Tohoku J. Exp. Med., this volume, p. 319.

2) The writers are indebted to Mr. Y. Amano in the Bacteriological Department for the ultracentrifugal examination.

3) Masamune & Yosizawa, Tohoku J. Exp. Med., 1957, **65**, 169.

4) Masamune & Kawasaki, *ibid.*, 1956, **63**, 369.

5) Gornall, Bardawill & Dâvid, J. Biol. Chem., 1948, **177**, 751.

6) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., Tohoku J. Exp. Med., this volume, p. 309.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

"Toxohormones" without KIK-potency from Urines, Cancerous and Normal—Preliminary Note

By

Hajime Masamune, Sen-itiroh Hakomori, Hirosi Kaketa
(正宗 一) (箱守 仙一郎) (掛田 博)

and **Sin-iti Abe**
(阿部 辰一)

Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, March 1, 1958)

Kaketa fractions[1], obtained by a modification of Nakagawa and Kasai procedure[2], of united cancerous urines with original seat in stomach, liver, lung or mamma and of normal urine were employed separately. The material was dissolved in slightly alkaline water and precipitated by acidulation with acetic acid. The precipitate was liberated from most of an urinary poison by treatment with Dowex 1 and electrodialysis, and the deposit occurring on the electrodialysis was added to a column of Dowex 1 in borate form and chromatographed with the same buffer to isolate the main component in an electrophoretically homogeneous state. Average yields of the cancerous and normal products amounted to 50 and 34 mg. per liter of urine respectively. Those products were both soluble in water at other pH values than 4.4–4.6, and free from phosphorus and proved to be glucidamins. The former product: N 13.5%, hexosamine 3.0%, galactose 1.6%, glucose 2.0%, mannose 1.5%, L-fucose 0.4%, sialic acid 3.4%, ash 0%, $[\alpha]_D^{\gamma} = -110°$; the latter product: N 13.8%, hexosamine 2.9%, galactose 1.9%, glucose 1.0%, mannose 1.4%, L-fucose 0.5%, sialic acid 4.4%, ash 0.6%, $[\alpha]_D^{\gamma} = -100°$. They proved both to be toxohormones, with the minimum active dosis per mouse of 1 mg. in the former and with that of 5 mg. in the latter. They lack KIK- and group-potency.

References: 1) Kaketa, Tohoku J. Exp. Med., this volume, p. 319. 2) Nakagawa & Kasai, Nippon Naikagakkai Zasshi (Jap.), 1951, **40**, 312.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

"Infarctoid Cardiopathy" Elicited by Temporary Obstruction of the Pylorus in Humorally Conditioned Animals

By

Hans Selye

From the Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Canada

(Received for publication, January 13, 1958)

It has been noted a short time ago that, in rats conditioned by NaH_2PO_4 for the cardiotoxic effects of certain corticoids, a brief treatment with the latter elicits a syndrome of acute, fatal, cardiac insufficiency, due to the development of large, infarct-like, necrotic patches in the myocardium. Among all the steroids tested, 2α-methyl-9α-chlorocortisol (Me-Cl-COL) proved to be the most effective in this respect, but a number of other highly active corticoids possess similar effects.[1),2)] It is particularly noteworthy that, in rats pretreated with otherwise ineffective, small doses of Me-Cl-COL plus sodium phosphate, sudden exposure to various stressors, such as a brief period of neuromuscular exertion[2)], bilateral vagotomy, hot or cold baths, or quadriplegia (induced by transecting the motor nerves of all four extremities) can precipitate the development of massive myocardial necroses[4)]. Still, this cardiotoxic action in humorally conditioned animals is not entirely non-specific, since some stressors (e.g., spinal cord transection, toxic doses of vasopressin) do not appear to possess it.[4)]

It is well known that symptoms of acute indigestion often precede death from cardiac infarcts in man. Hence, we have now performed experiments, in which a temporary occlusion of the pylorus was produced (in rats suitably conditioned by in themselves inactive, threshold doses of Me-Cl-COL plus NaH_2PO_4), in order to determine whether the resulting transient gastrointestinal disturbance can also act as a precipitating agent for cardiac death.

Experimental

Materials and Techniques

Sixty female Sprague-Dawley rats, with an average initial body-weight of 123 g. (range: 115–130 g.), were subdivided into three equal groups, as indicated in Table I. The animals of Groups I and III received 2α-

methyl-9α-chlorocortisol (Me-Cl-COL) in the form of a microcrystal suspension of its acetate, at the dose of 50 μg. in 0.2 m*l*. of water, once daily subcutaneously. At the same time, they were also given monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$), at the dose of 300 mg. in 4 m*l*. of water, twice daily, by stomach tube.

Forty-eight hours after initiation of this treatment, the pylorus was temporarily ligated for one hour in all the rats of Groups II and III. The following technique was used:

1) the animal is shaved in the upper epigastric region, anesthetized with ether and fastened to an operating board in the supine position;

2) the pylorus is exteriorized through a midline incision and, after blunt separation of the adherent pancreas, the duodenum next to the pylorus is placed into the lumen of a piece of slit-open rubber tubing (length: 7 mm., diameter: 5 mm.);

3) the duodenum now enveloped in its protective rubber covering is completely but gently compressed by a nylon ligature, and the abdominal wound is closed. (The purpose of shielding the duodenum with the rubber tubing is to prevent any excessive local injury.);

4) after the operation, the animals are allowed to awaken and, one hour later (again under ether anesthesia), the wound is opened and the pylorus is rendered permeable again by transecting the nylon thread and removing the rubber tubing.

This operation was well tolerated by all the animals, although the usual daily treatment with Me-Cl-COL and NaH_2PO_4 continued after the intervention. However, during the subsequent days, 50% of these rats succumbed with manifest signs of infarctoid cardiopathy.

Throughout the period of observation, the animals were fed exclusively on the laboratory diet, " Purina Fox Chow," *ad libitum*.

The experiment was terminated 96 hours after initiation of treatment—that is, 48 hours following the temporary pyloric obstruction—by killing all survivors with chloroform. Immediately after autopsy, the heart and kidneys were fixed in neutral formalin for subsequent staining with hematoxylin-phloxine (to determine the general histologic structure), and with von Kossa's silver nitrate technique (for the histochemical demonstration of calcium). The kidneys were examined because our earlier work had revealed the common occurrence of nephrocalcinosis in rats treated simultaneously with NaH_2PO_4 plus Me-Cl-COL[3]. The incidence (per cent positive) and grade (estimated in terms of an arbitrary scale of 0 to 3) of both the cardiac necrosis and the nephrocalcinosis are listed, together with the mortality rate, in Table I. To appraise the significance of the differences in the severity of the lesions, the standard errors are also given.

Results

Even prior to the termination of the experiment, it was obvious that a temporary occlusion of the pylorus—which is well tolerated by otherwise untreated rats—is extremely damaging to those conditioned by Me-Cl-COL plus NaH_2PO_4, since, among the latter animals, 50% died with macroscopically visible, large, whitish patches of cardiac necrosis. The autopsy findings are summarized in Table I.

TABLE I

" Infarctoid Cardiopathy " Elicited by Temporary Obstruction of the Pylorus in Humorally Conditioned Animals

Group	Treatment	Cardiopathy		Nephrocalcinosis		Mortality (%)
		Incidence (%)	Grade (Scale: 0–3)	Incidence (%)	Grade (Scale: 0–3)	
I	Me-Cl-COL + NaH_2PO_4	10	0.2±0.1	90	1.5±0.25	0
II	Pylorus ligature	0	0	0	0	0
III	Me-Cl-COL + NaH_2PO_4 + Pylorus ligature	100	2.9±0.2	100	2.8±0.3	50

The brief conditioning treatment with Me-Cl-COL plus NaH_2PO_4 caused macroscopically detectable evidence of cardiac necroses only in two out of the 20 rats in Group I, and even here the lesions were minimal. The nephrocalcinosis, on the other hand, was fairly pronounced, in 90% of the rats in this group. The animals subjected only to pylorus ligature, without previous Me-Cl-COL plus NaH_2PO_4 treatment (Group II), showed no evidence of cardiopathy or nephrocalcinosis. On the other hand, in the group which was subjected to temporary occlusion of the pylorus, after conditioning by Me-Cl-COL plus NaH_2PO_4 (Group III), multiple, extensive patches of myocardial necroses could be detected by naked-eye inspection in every animal. The nephrocalcinosis was likewise constant and significantly more intense in this than in the previous group.

It is evident from these observations that a threshold dose of Me-Cl-COL plus NaH_2PO_4—which in itself causes no cardiopathy in the vast majority of the animals and only a moderate degree of nephrocalcinosis—conditions the animals so that they all respond with severe myocardial necroses and nephrocalcinosis to a temporary occlusion of the pylorus.

Histologic study merely confirmed the macroscopic observations. The whitish patches in the myocardium proved to consist of necrotic muscle fibers, which were gradually removed by monocytes and replaced by connective tissue (Fig. 1). In the kidney, silver-nitrate-tingible calcium deposits were found with particular frequency in the convoluted tubules of

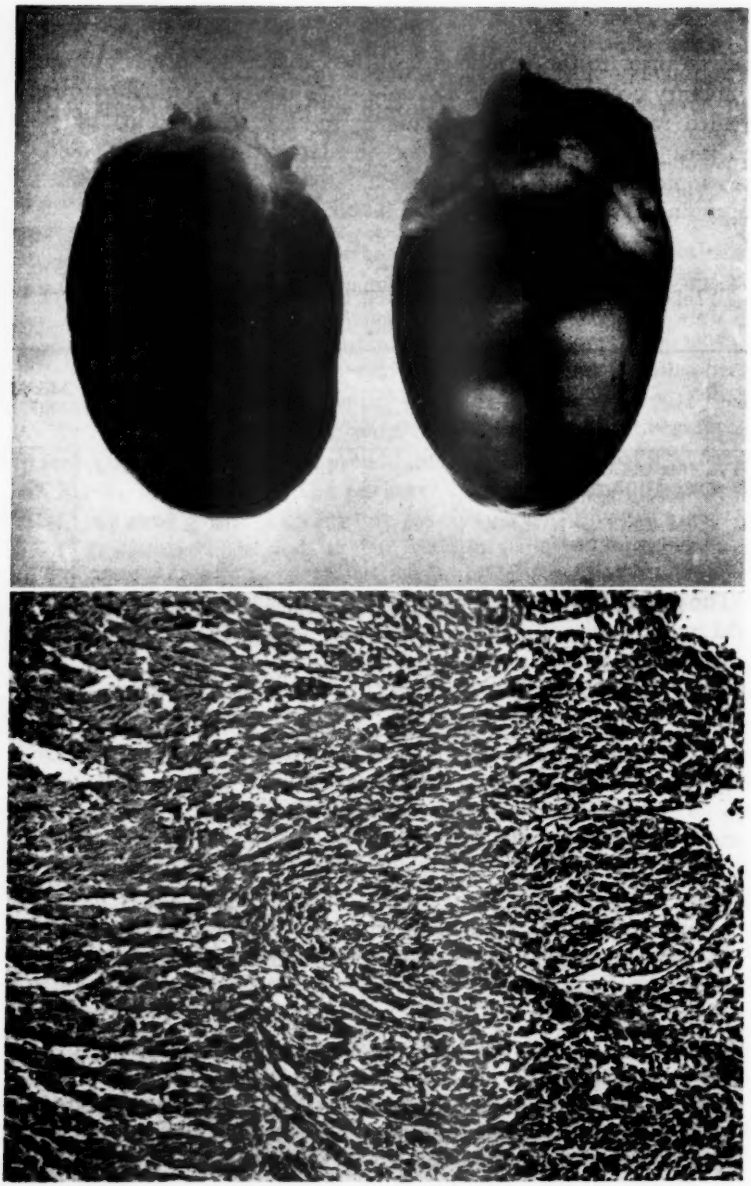


Fig. 1. *Top*: Normal appearance of the ventricle in a rat treated with Me-Cl-COL plus NaH_2PO_4 (Group I) on the left, in comparison with the heart of a similarly treated rat in which temporary occlusion of the pylorus has elicited multiple, massive, whitish necrotic patches (Group III).

Bottom: Histologic appearance of one of the infarcted regions in the heart shown above on the right. The normal muscle on the left side of the microscopic field is sharply demarcated from the necrotic, subendocardia', infarctoid patch on the right (hematoxylin-phloxine × 100).

the cortex, especially at the corticomedullary junction line.

In these experiments it is not possible to distinguish clearly between the specific actions of pylorus obstruction and the nonspecific stressor effect of the operative intervention. In several of the animals in Groups II and III, inflammatory reactions and adhesions around the pylorus resulted in a continued retention of gastric juice within the stomach, even after release of the ligature. It is known that prolonged pyloric stenosis (perhaps as a consequence of the resulting alkalosis) may in itself cause some nephrocalcinosis and occasionally, myocardial necroses[5]. Still, in most of the animals of Group III (humorally conditioned), the pylorus was perfectly permeable and nevertheless the cardiopathy and the nephrocalcinosis were severe while, in the rats of Group II (not conditioned), which were submitted to the same surgical procedure, no trace of cardiopathy or nephrocalcinosis resulted.

In any event, the observations clearly demonstrate that selective damage to the upper intestinal tract is highly effective in eliciting massive myocardial necroses in rats conditioned for this effect by treatment with a potent corticoid and sodium phosphate. The infarctoid cardiopathy thus produced differs from true cardiac infarcts, in that it is not occasioned by any microscopically detectable coronary occlusion; yet, our observations suggest that, following humoral conditioning, the cardiac musculature can become highly susceptible to the production of massive necroses by damage to the gastrointestinal tract. This fact should be kept in mind in evaluating the possible pathogenetic role of acute gastrointestinal irritation in the production of cardiac infarcts in predisposed patients.

Summary

In rats pretreated with 2α-methyl-9α-chlorocortisol (Me-Cl-COL) and NaH_2PO_4, temporary occlusion of the pylorus regularly elicits acute, massive myocardial necroses and nephrocalcinosis, as well as a high mortality. The possible clinical implications of this fact, as regards the pathogenetic role of acute gastrointestinal disorders in the production of cardiac infarcts, is discussed.

Acknowledgements

These investigations were supported by grants from the Gustavus and Louise Pfeiffer Research Foundation and the National Heart Institute of the U. S. Department of Health and Welfare.

The author gratefully acknowledges generous supplies of 2α-methyl-9α-chlorocortisol from The Upjohn Company.

References

1) Selye, H., Discussion. Laurentian Hormone Conf., Mont-Tremblant, 1957, Sept. 1–6.
2) Selye, H. & Mishra, R. K., Amer. Heart J., 1958, in press.
3) Selye, H. & Renaud, S., Proc. Soc. Exper. Biol. and Med., 1957, **96**, 512.
4) Selye, H., Renaud, S. & Nádasdi, M., Endocrinology, 1958, in press.
5) Selye, H. & Ledoux, R., Gastroenterology, 1958, in press.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Studies on Leucocytosis and Leucopenia in Infectious Diseases

Report III

On the Mitosis of Nucleated Cells in the Bone Marrows of Leucocytosis and Leucopenia

By

Akira Saito and **Tokuji Miyamoto**
(斎藤　章)　　(宮本篤次)

From the Medical Clinic of Prof. T. Kurokawa, Tohoku University Sendai

(Received for publication, January 15, 1958)

As mentioned in the first[1] and second[2] reports, in the hematopoiesis of the bone marrow in infectious diseases there is a great difference between leucocytosis and leucopenia. This difference seems to be caused originally by the mitosis in the bone marrows.

Concerning mitosis of the nucleated cells in the bone marrows of normal persons and patients suffering from blood diseases, first Ernst[3] and then Lindenbaum[4], Segerdahl[5], Picena[6], Osgood[7], Müllendorf[8] and Henning[9] have reported: Rohr & Schudel[10] have gone into details, but few have been the reports on mitosis of the bone marrows in infectious diseases. The authors have observed mitosis of the nucleated cells in the bone marrows by serial punctures following recovery, in order to seek the causes of the difference between leucocytosis and leucopenia in infectious diseases.

Experimental

Methods

In our examinations 5 cases of leucocytosis (4 cases of Japanese encephalitis and one of scarlet fever) and 5 cases of leucopenia (3 typhoid and 2 paratyphoid cases) were studied for the mitotic patterns of the nucleated cells in the bone marrows, obtained by serial punctures during the various stages of the diseases, using 5 normal persons as controls. The bone marrow and the blood specimens were taken by the same procedure discussed in the first and the second reports. The bone marrow punctures of all patients were done regularly in three stages of the acme, the fever-abating stage and the reconvalescence. The peripheral blood was observed every third day. The nucleated cells in the bone marrow were classified according to Rohr's method, and divided into the 3 systems:

reticuloendothelial, erythropoietic and leuco- and lymphopoietic. The mitotic cells in every system were counted, which appeared in the field of vision during the counting of 10,000 cells in the leuco- and lymphopoietic system and the mitosis index was calculated.

Results

1) Mitosis of the nucleated cells in the bone marrows of normal persons

Table I represents the mitosis index of the nucleated cells in the bone marrows of normal persons. In the *erythropoietic system* the mitosis index of basophil erythroblasts is 93.3‰, of polychromatic erythroblasts 39.3‰, of oxyphil erythroblasts 29.2‰, while in the *leucopoietic system* the mitosis index of myeloblasts is 3‰, of unmatured myelocytes 8.3‰, of half-matured myelocytes 18.4‰, of matured myelocytes 7‰ and of eosinophil cells 2.9‰. That is, the mitotic function is most accelerated in half-matured myelocytes. In normal persons, the mitosis index of the nucleated cells in the bone marrows amounted to 41.8‰ in the erythropoietic system and to 2.4‰ in the leucopoietic system.

TABLE I

Mitosis Index in Normal Cases

Kind of cell	Erythropoiesis			Leucopoiesis				
	Erythroblasts			Myeloblasts	Neutrophil myelocytes			Eosinophils
	Basophil	Polychromatic	Oxyphil		Unmatured	Half-matured	Matured	
Cell counts	1660	7710	5460	670	1560	2990	4740	2450
Mitosis counts	155	304	162	2	13	55	33	7
M. I. ‰	93.3	39.3	29.2	2.99	8.33	18.39	6.98	2.86

It seems to be better for easy understanding that the frequency of mitosis appearing in the microscopic fields of vision be shown in percentages. Table II shows the frequency of mitosis in the bone marrow of normal persons in percentages. In the erythropoietic system the mitosis counts were 24% in basophil erythroblasts, 48.4% in polychromatic erythroblasts and 27.5% in oxyphil erythroblasts. In the leucopoietic system the mitosis counts were 1.6% in myeloblasts, 10.8% in unmatured myelocytes, 45.7% in half-matured myelocytes, 36.1% in matured myelocytes and 5.8% in eosinophil cells. That is to say, the frequency of mitosis both in the erythropoietic and leucopoietic system showed the highest percentage in half-matured cells followed by matured cells.

The frequency of the 4 phases in mitosis. All the mitotic figures were divided into 4 phases: prophase, metaphase, anaphase and telophase. Showing the frequency of every phase in percentage, both in the erythropoietic and the leucopoietic systems the frequency in these phases was highest in prophase, followed by metaphase, telophase and anaphase in the order named, as shown in Table III. The ratio of these percentages is proportionate to the ratio of durations of these phases.

TABLE II

Frequency of Mitosis in Normal Cases

Erythropoiesis				Leucopoiesis						
M. I. ‰	Erythroblasts			M. I. ‰	Myeloblasts %	Neutrophil myelocytes			Metamyelocytes %	Eosinophils %
	Basophil %	Polychromatic %	Oxyphil %			Unmatured %	Half-matured %	Matured %		
38.5～46.3	18.6-33.2	42.1-53.9	12.9-39.3	2.2～2.7	0-4.3	4.6-16.6	39.2-58.2	25.0-54.6	0	0-13.1
41.84	24.04	48.44	27.52	2.4	1.60	10.80	45.74	36.08	0	5.78

TABLE III

Frequency of Every Mitosis-Phase in Normal Cases

System	Erythropoietic system				Leucopoietic system			
Phase	Pro.	Meta.	Ana.	Telo.	Pro.	Meta.	Ana.	Telo.
%	46.6-67.6	8.8-22.9	9.1-14.3	11.8-18.2	48.2-66.6	12.5-25.8	4.6-14.8	8.4-18.2
Mean value %	54.42	18.66	12.12	14.80	55.12	20.66	10.68	13.52

In the *reticuloendothelial system* mitosis was seen chiefly in plasma cells, observed in about 1‰ of them. Beside mononucleated cells, two-, three- or four-nucleated plasma cells appeared not seldom in the bone marrow. These polynucleated plasma cells could not be determined whether they had proliferated by mitosis or by amitosis.

2) Mitosis of the nucleated cells in the bone marrow of leucocytosis

Mitosis in the various stages of the illness in leucocytosis.

Mitosis of the nucleated cells in the bone marrow was observed in the cases of leucocytosis. These cases had been already in the peak stage of the illness when they were admitted to our hospital, therefore mitosis was observed in the acme, the fever-abating stage and the reconvalescence, but not in the fever-rising stage. Table IV shows the mitosis index in *the erythropoietic system of leucocytosis*. The mitosis index in the erythropoietic system showed about the normal value of 42‰, and the red blood cell count in the blood stood at the normal level of 4.52-4.78 millions. In the bone marrow, the mitosis index was 26.9‰ in basophil erythroblasts, the highest of 28.2‰ in polychromatic erythroblasts and 15‰ in oxyphil erythroblasts in the peak stage of the illness, as listed in Table V, showing the highest mitosis index in basophil erythroblasts as in normal cases after the fever-abating stage.

The frequency of mitosis in the erythropoietic system in the various stages of the illness is shown in Table VI. The frequency of mitosis in polychromatic

TABLE IV

Relation Between Mitosis Index in the Erythropoietic System and the Red Blood Cell Count in the Peripheral Blood in Every Stage of the Illness

Disease	Leucocytosis			Leucopenia		
Stage of the illness	Acme	Fever-abating	Reconv.	Acme	Fever-abating	Reconv.
M. I. ‰	38.7–47.0	4.02–44.2	38.2–47.2	35.9–48.4	35.5–50.5	38.6–58.1
Mean value ‰	41.46	42.45	42.20	42.28	42.74	45.88
R. B. C. (Million)	3.53–5.44	3.88–5.39	4.00–5.31	2.64–4.15	2.98–4.48	3.66–4.72
Mean value (Million)	4.52	4.59	4.78	3.35	3.85	4.33

TABLE V

Mitosis Index in Various Kinds of Cell in the Erythropoietic System in Every Stage of the Illness

Disease		Leucocytosis			Leucopenia		
Stage	Kind of cells→	Basophil	Polychromatic	Oxyphil	Basophil	Polychromatic	Oxyphil
Acme	Cell counts	1.187	3.770	3.602	1.036	2.740	5.108
	Mitosis counts	32	106	54	66	99	79
	M. I. ‰	26.9	28.2	15.0	63.8	36.1	15.4
Fever-abating	Cell counts	578	2294	1.238	1.456	3.710	3.912
	Mitosis counts	17	51	19	54	96	72
	M. I. ‰	29.5	23.6	15.4	37.1	25.8	14.7
Reconvalescence	Cell counts	1.374	3.396	4.310	1.139	3.740	4.368
	Mitosis counts	45	108	65	46	107	68
	M. I. ‰	32.7	31.9	15.1	40.3	28.6	15.5

TABLE VI

Frequency of Mitosis in the Erythropoietic System in Every Stage of the Illness (‰)

Disease	Leucocytosis			Leucopenia		
Kind of cell→ / Stage ↓	Basophil	Polychromatic	Oxyphil	Basophil	Polychromatic	Oxyphil
Acme	16.6	55.2	28.2	27.1	40.6	32.3
Fever-abating	19.5	58.7	21.8	24.4	43.4	32.2
Reconvalescence	20.6	49.6	29.8	20.8	48.5	30.7

erythroblasts showed the highest percentage in every stage of the illness as in normal bone marrows.

In *the leucopoietic system* the mitosis index amounted to 3.28‰ (2.6–4.3‰) and was apparently more accelerated than in normal cases in the peak stage, when the leucocyte count was 11,440 in the peripheral blood, then it lowered frankly to a somewhat subnormal value of 2.3‰ (1.8–3.2‰) in the fever-abating stage, but it rerose in the reconvalescence, showing about the normal value of 2.58‰ (1.8–3.3‰), when the leucocyte count returned to the normal level of 8,360 in the blood (Table VII).

TABLE VII

Relation between the Mitosis Index in the Leucopoietic System and the White Blood Cell Count in the Peripheral Blood in Every Stage of the Illness

Disease	Leucocytosis			Leucopenia		
Stage	Acme	Fever-abating	Reconv.	Acme	Fever-abating	Reconv.
M. I. ‰	2.6–4.3	1.8–3.2	1.8–3.3	1.6–2.4	1.7–3.8	2.1–2.4
Mean value ‰	3.28	2.30	2.58	1.94	2.52	2.26
W. B. C.	9.400–13.300	7.400–12.000	7.000–9.700	2.600–7.000	2.500–6.300	3.300–6.800
Mean value	11.440	9.400	8.360	4.400	4.840	5.540

It is a remarkable finding of confusions in mitosis in the case of severe Japanese encephalitis that in the leucopoiesis of the bone marrow irritation and inhibition take place at the same time during the acme and the fever-abating stage; as a result of the former, a nuclear shift to the right in the bone marrow and a nuclear shift to the left in the blood, but on the contrary, as a result of the latter, appearance of overripened neutrophil and eosinophil cells could be observed both in bone marrow and blood.

Table VIII shows the mitosis index in the various kinds of the nucleated cells in the leucopoietic system. Mitosis was accentuated strikingly in the various kinds of cells and the mitosis index showed the highest value of 26.9‰ in the half-matured myelocytes in the peak stage, but after that mitosis gradually lowered and in the course of the illness the mitosis index of unmatured myelocytes and myeloblasts was accelerated while that of matured myelocytes was lowered. Thus mitosis centered on the left of the bone marrow. That is to say, in the course of the illness inhibition followed irritation physiologically in leucopoiesis of the bone marrow. The frequency of mitosis in the leucopoietic system in every stage of the illness is shown in Table IX. In the peak stage mitosis showed the highest percentage of 43% in matured myelocytes, followed by 35.2% in half-matured myelocytes. Mitosis centered upon the half-matured myelocytes in the defervescence and in the convalescence, mitosis first leaning over to the half-matured and unmatured myelocytes and finally over to the half-matured myelocytes, as in a normal person.

In the peripheral blood a nuclear shift to the left was remarkable in the peak stage, when mitosis centered upon the matured myelocytes and a nuclear

TABLE VIII

Mitosis Index in Various Kinds of Cells in the Leucopoietic System in Every Stage of the Illness

Disease		Leucocytosis					Leucopenia				
Stage	Kind of cell	Myeloblasts	Myelocytes: Unmatured	Myelocytes: Half-matured	Myelocytes: Matured	Eosinophils	Myeloblasts	Myelocytes: Unmatured	Myelocytes: Half-matured	Myelocytes: Matured	Eosinophils
Acme	Cell counts	607	1.175	2.152	4.227	2.602	580	853	1.697	4.118	1.389
	M. counts	9	23	58	71	4	5	17	35	37	4
	M. I. ‰	14.8	19.6	26.9	16.9	1.5	8.6	19.9	20.6	9.1	2.8
Fever-abating	Cell counts	378	914	1.690	2.478	1.592	428	1.242	1.708	3.978	992
	M. counts	2	14	32	30	12	6	20	50	44	4
	M. I. ‰	5.4	15.2	19.0	12.2	7.6	14.0	16.0	29.2	11.0	4.0
Reconvalescence	Cell counts	773	1.215	2.294	4.079	6.111	446	932	1.636	3.526	1.266
	M. counts	5	25	59	43	25	4	30	50	40	4
	M. I. ‰	6.5	20.5	25.8	10.5	4.1	9.0	32.2	30.6	12.6	3.2

TABLE IX

Frequency of Mitosis in the Leucopoietic System in Every Stage of the Illness (%)

Kind of cell	Leucocytosis						Leucopenia					
	Myeloblasts	Myelocytes: Unmatured	Myelocytes: Half-matured	Myelocytes: Matured	Metamyelocytes	Eosinophils	Myeloblasts	Myelocytes: Unmatured	Myelocytes: Half-matured	Myelocytes: Matured	Metamyelocytes	Eosinophils
Acme	5.4	14.0	35.2	43.0	0	2.4	5.6	17.4	35.7	37.8	0	3.5
Fever-abating	2.2	15.6	35.6	33.3	0	13.3	4.8	16.2	40.3	35.4	0	3.3
Reconvalescence	3.9	15.9	37.6	27.4	0	15.9	1.7	23.2	39.3	32.2	0	3.6

right shift appeared in the bone marrow, while a nuclear right shift took place gradually in the blood in the reconvalescence, when a nuclear left shift was seen in the bone marrow.

As shown in Table VIII and IX, it was noteworthy that the mitosis index and the frequency of mitosis in neutrophil cells in the bone marrow dropped subnormally in the fever-abating stage. It is because the various kinds of cells had increased in number on one hand, and mitosis of eosinophil cells, which accounted only for 1.5‰ (2.4%) in the peak stage, rose remarkably in the fever-

abating stage to 7.6‰ (13.3%), falling again in the reconvalescence 4.1‰ (15.9%), on the other. In the fever-abating stage and the reconvalescent stage mitosis of eosinophil cells was 2–3 times higher than in normal persons.

In the peripheral blood eosinophils decreased remarkably or disappeared from the blood in the peak stage of Japanese encephalitis cases but after the fever-abating stage they increased gradually and showed a postinfectious eosinophilia.

Mitosis in the reticuloendothelial system. The mitosis index in plasma cells rose to the highest value of 2.16‰ in the peak stage, which was higher than normal, but in the course of the illness dropped gradually, showing 1.32‰ in the fever-abating stage and 1.45‰ in the reconvalescent stage (Table X).

TABLE X

Mitosis Index in the Reticuloendothelial System in Every Stage of the Illness

Disease	Leucocytosis			Leucopenia		
Stage of the illness	Acme	Fever-abating	Reconv.	Acme	Fever-abating	Reconv.
M. I. ‰	0–4.5	0–3.2	0–3.5	0–2.0	0–2.9	0–2.4
Mean value ‰	2.16	1.32	1.45	1.08	0.98	1.24

TABLE XI

Frequency of Every Mitosis-Phase in the Erythropoietic System (%)

Disease	Leucocytosis				Leucopenia			
Phase→	Pro.	Meta.	Ana.	Telo.	Pro.	Meta.	Ana.	Telo.
Acme	40.7	18.7	13.0	27.6	39.5	22.5	13.1	24.9
Fever-abating	41.6	25.3	12.6	20.5	44.6	25.2	12.6	17.6
Reconvalescence	38.6	19.2	14.6	27.6	44.4	21.2	14.5	19.9

TABLE XII

Frequency of Every Mitosis-Phase in the Leucopoietic System (%)

Disease	Leucocytosis				Leucopenia			
Phase→	Pro.	Meta.	Ana.	Telo.	Pro.	Meta.	Ana.	Telo.
Acme	57.6	17.0	17.5	7.9	22.5	42.8	27.6	7.1
Fever-abating	55.5	6.7	6.7	17.8	38.7	33.9	16.2	11.2
Reconvalescence	51.7	15.3	15.3	8.9	57.2	25.0	14.3	3.5

In the frequency of 4 phases of mitosis in the erythropoiesis and leucopoiesis there is no great difference between leucocytosis cases and normal cases (Table XI).

3) Mitosis of the nucleated cells in the bone marrow of leucopenia.

Mitosis in the various stages of the illness in leucopenia.

Mitosis of the nucleated cells in the bone marrow was observed in 5 cases of leucopenia (3 typhoid fever and 2 paratyphoid A cases). The mitosis index *in the erythropoietic system of leucopenia* cases was 42.3‰ in the peak stage, 42.7‰ in the defervescent stage and 45.9‰ in the reconvalenscent stage (Table IV). That is, mitosis index was accelerated in the later course of the illness. The red cell count in the periphery amounted to 3.35 millions in the peak stage, to 3.85 millions in the defervescent stage and to 4.33 millions in the reconvalescent stage. Therefore the red cell count in the periphery ran parallel to the mitosis index of erythroblasts in the bone marrow, as presented in Table IV.

The mitosis index showed the highest value in basophil erythroblasts in the bone marrow in every stage, as shown in Table V. The frequency of mitosis in the erythropoietic system in the various stages of the illness is shown in Table VI, the frequency of mitosis in polychromatic erythroblasts showed the highest percentage in every stage of the illness as in normal cases.

In *the leucopoietic system of leucopenia* there is a characteristic drop in mitosis, as Table VII shows. The mitosis index was subnormal value of 1.94‰ (1.6–2.4‰), showing a decided drop of the mitosis index in matured myelocytes in the peak stage, when the white cell count in the periphery was 4,400 (2,600–7,000).

In the defervescent stage the mitosis index in the leucopoietic system rose somewhat supernormally to 2.52‰ (1.7–3.8‰), because the various kinds of cells had decreased remarkably in the bone marrow, while in the peripheral blood the leucocyte count showed a subnormal level of 4,840 (2,500–6,000) and a rise or drop of mitosis in the bone marrow was followed by a similar change in the blood about 4–7 days afterwards.

In the reconvalescent stage, the mitosis index showed about the normal value of 2.26‰ (2.1–2.4‰), the leucocyte count increasing to a nearly normal value of 5,540 (3,300–6,800) in the blood.

Table VIII shows the mitosis index in the various kinds of the nucleated cells in the leucopoietic system of leucopenia. The mitosis index in the various kinds of cells in the bone marrow rose relatively higher than the normal in the peak stage, showing the highest value of 20.6‰ in half-matured myelocytes, because of the decrease in their absolute count. The mitosis index rose more relatively in half-matured and matured myelocytes in the defervescence and in unmatured myelocytes rose over that of half-matured myelocytes in the reconvalescent stage.

The frequency of mitosis in the leucopoietic system of the bone marrow showed the highest percentage of 37.8% in matured myelocytes in the peak stage, that of 40.3% in half-matured myelocytes in the defervescent stage but in the reconvalescent stage the frequency of mitosis in unmatured myelocytes remarkably rose to the percentage of 23.2%. Therefore a drop of mitosis appeared remarkably in the half-matured and matured myelocytes in the Peak Stage, and then mitosis recovered from the left to the right in the bon marrow (Table IX).

In leucopenia it is a characteristic in mitosis of the leucopoietic system that after the peak stage the mitosis count in every kind of cells increased in the course of the illness. The mitosis index in *the reticuloendothelial system* was about 2–1‰, and showed nothing characteristic, as shown in Table X.

The frequency of the 4 phases of mitosis observed in the erythropoietic system in leucopenia was similar to that seen in normal persons (Table XI, XII) but that in the leucopoietic system showed the highest percentage of 42.8% in metaphase, followed by anaphase, prophase and telophase in the order named, in the peak stage, when mitosis showed the minimal value, but it returned to a nearly normal proportion after the reconvalescent stage, as presented in Table XII.

Differences between leucocytosis and leucopenia observed from the standpoint of mitosis in the bone marrow

Comparing leucocytosis and leucopenia on the basis of mitosis in the bone marrow, we find the mitotic function in the erythropoietic system accelerated most in basophil erythroblasts, but the frequency of mitosis rising highest in polychromatic erythroblasts both in leucocytosis and leucopenia. The frequency of mitosis in polychromatic erythroblasts was 50–59% in leucocytosis and 40.6–48.5% in leucopenia. It is particularly noteworthy that the mitosis index in the erythropoietic system remained relatively low in the case with a very acute but short course, such as encephalitis japonica. On the contrary, the mitosis index rose strikingly in the acme and defervescent stage of a severe case with a prolonged course and anemia of about 3,00 millions, such as typhoid fever. In the erythropoietic system the mitosis index was intimately related to anemia, which was proportionate to the destruction of erythrocytes in the periphery and the severity of the illness, and consequently when after the peak stage anemia was the more remarkable the more the mitosis index rose in the bone marrow, but both in leucocytosis and leucopenia there was no basic difference in mitosis of the erythropoietic system.

On the other hand in mitosis of the leucopoietic system there was, however, a decided difference between the two. In leucocytosis mitosis was accentuated and the mitosis index rose to 3.28‰ in the peak stage, when in leucopenia it lowered markedly and the mitosis index was only 1.94‰, therefore the mitosis index in the former was about 2 times higher than that in the latter. From the defervescence mitosis dropped in the former and rose in the latter, attaining the normal level after the reconvalescence.

Intervals in which the rise and drop of mitosis in the bone marrow were projected on the peripheral blood

In the bone marrow a rise or drop in mitosis caused an increase or a decrease of the nucleated cells, and was followed by a similar change in the peripheral blood about 4–7 days afterwards, as shown in Table XIII, both in leucocytosis and leucopenia.

In general there was a constant relation between the mitosis index in the bone marrow and the white blood cell count in the peripheral blood in infectious diseases both in leucocytosis and leucopenia. If the leucocyte level of the peri-

TABLE XIII

Intervals, in which a Rise or Drop of Mitosis in the Bone Marrow was Projected on the Peripheral Blood

Examinated at the same time		Intervals	After the intervals
W. B. C.	M. I. (‰)	Days	W. B. C.
2.500	2.2	8	3200
2.600	2.0	10	2300
3.200	1.6	6	2800
3.300	2.2	7	5400
4.400	1.8	7	5200
4.800	2.8	7	5400
5.200	2.0	5	5800
5.400	1.7	6	5200
5.600	2.1	7	7000
5.800	2.3	7	6800
6.200	2.3	4	6200
6.300	2.1	4	6500
6.800	2.4	4	8900
7.000	2.4	7	6800
7.000	2.4	7	8800
7.400	1.8	7	7000
8.800	2.5	5	9000
9.000	3.2	6	9600
9.200	1.9	7	9000
10.100	4.3	4	10.600
12.200	2.9	8	9200
12.200	2.6	4	9000
13.300	2.7	7	9200

pheral blood can be divided into the three groups of less than 6,000 (subnormal), 6,001–9,000 (normal) and more than 9,001 (supernormal), the mitosis index in the leucopoietic system stood at 2.17‰ in subnormal, at 2.5‰ in normal and at 3.2‰ in supernormal cases, as shown in Table XIV. That is to say, the white blood cell counts in the peripheral blood ran parallel to the mitosis index in the bone marrow.

TABLE XIV

Relation between Mitosis Index and the White Blood Cell Counts in the Peripheral Blood

W. B. C.	2001—6.000	6.001—9.000	9.001—13.000
Disease	Leucopenia	Normal	Leucocytosis
M. I. ‰	1.90—2.38	2.20-3.10	2.60-4.30
Mean value ‰	2.17	2.50	3.20

It is a noteworthy fact that the nucleated cell count in the bone marrow usually showed a considerable variation, and it varied greatly besides in the cases the bone marrow was punctured by an unskilled operator: too much aspiration and the needle was stuck into large sinus of the bone marrow. It

may be said, however, in the cases of leucocytosis the bone marrow was in general rich in nucleated cells while in the cases of leucopenia it was poor in them, shown as in Table XV and XVI. The highest nucleated cell count in the bone marrow was 350,000 in the former but it was only 180,000 in the latter. Therefore, the nucleated cell count in the former not rarely reached the level about twice or three times of that in the latter.

Comments

The frequency of mitosis in the erythropoietic system is 41.8‰ in our examination of 5 normal persons. Concerning the normal mitosis index in the erythropoietic system Picena[6] reported 1–2‰ as normal, Segerdahl[5] reported 11.7‰ in males and 18.3‰ in females, Fiesch[11] found a Similar value of about 12–20‰. These results are lower than the ours. Rohr[10] reported the value of 42‰, which is nearly equal to our result. In our examination the normal mitosis index in the leucopoietic system is 2.4‰ (2.2–2.7‰) which is nearly equal to Rohr's report, but Picena[6] states 1–2‰ as normal. Segerdahl[5] reported 0.69‰ in myelocytes and 0.29‰ in myeloblasts, Fiesch 1‰ in leucopoiesis with variations up to 5–6‰, in exceptional cases to 10‰.

In the reticuloendothelial system mitosis was seen chiefly in plasma cells and observed in about 1‰ which is equal to the value reported by Rohr. In the bone marrow large plasma cells with 2–5 nuclei were frequently observed, but it could not be determined whether they prolifereted by mitosis or by amitosis.

In mitosis of the erythropoietic system there is no essential difference between leucocytosis and leucopenia. In the erythropoietic system the mitosis index is intimately related with the clinical course. Therefore the mitosis index showed about a normal value in the majority of the cases of Japanese encephalitis, in which the clinical course was very short and on about the seventh day of the illness defervescence regularly sets in without remarkable anemia in the peripheral blood. It is because the clinical course is very short in comparison with the duration of life of the red blood cells. On the contrary in the case of typhoid fever with prolonged course a considerable anemia appeared in the blood and the mitosis index was accelerated more than the normal in order to compensate for it. Our results are similar to those in Rohr's report. He reported that the mitosis index in the erythropoietic system was accelerated in the case of anemia and the mitosis index was 66‰ in the case of anemia caused by bleeding and 120‰ in the case of hemolytic anemia.

In the leucopoietic system in the peak stage the mitosis index was accelerated strikingly and showed the maximal level of 3.28‰ in leucocytosis, while it lowered remarkably and showed the minimal level of

TABLE

Frequency of Every Phase of

Name, Age, Sex	Disease	Stage	Day of the illness	Temp. C°	Hgb. %	R.B.C. in B. (Million)	W.B.C. in B.	Nucleated cells in B. M.	Counted cells		M. counts	M. I. ‰
E.Y. 18 ♂	Enceph. jap.	Acme	7	39.9	72	3.53	12200	154000	Ret	526	0	0
									R	2134	42	39.3
									W	10000	26	2.6
		Fever-abating	11	37.4	81	4.21	9000	193000	Ret	286	0	0
									R	1070	21	42.3
									W	10000	32	3.2
		Reconv.	25	36.8	83	4.00	9700	237000	Ret	382	0	0
									R	2220	46	41.6
									W	10000	33	3.3
M.Y. 5J10M ♂	Enceph. jap.	Fever-abating	16	37.4	80	3.88	7400	351200	Ret	301	0	0
									R	794	17	42.7
									W	10000	18	1.8
		Reconv.	25	37.1	86	4.89	7000	181000	Ret	280	1	3.5
									R	1966	43	43.7
									W	10000	24	2.4
		Reconv.	39	36.5	93	5.31	9500	298000	Ret	402	1	2.5
									R	1544	33	42.7
									W	10000	33	3.3
S.J. 8J ♂	Enceph. jap.	Acme	7	38.6	95	5.44	13300	91000	Ret	437	2	4.5
									R	817	33	40.4
									W	10000	27	2.7
		Fever-abating	14	38.0	92	5.39	9200	99300	Ret	594	2	3.2
									R	890	19	42.7
									W	10000	19	1.9
		Reconv.	26	37.1	89	4.95	8800	142300	Ret	866	0	0
									R	1430	57	39.8
									W	10000	25	2.5
S.T. 19 ♀	Enceph. jap.	Acme	12	38.1	90	4.75	9400	138400	Ret	588	2	3.4
									R	2004	42	41.9
									W	10000	39	3.9
		Acme	41	37.9	90	4.15	12200	160200	Ret	690	2	2.9
									R	2326	45	38.7
									W	10000	29	2.9
		Fever-abating	55	37.6	89	4.89	12000	127400	Ret	472	1	2.1
									R	1356	30	44.2
									W	10000	23	2.3
S.T. 6 ♀	Scarlet fever	Acme	2	38·9	91	4.73	10100	125000	Ret	478	0	0
									R	1278	30	47.0
									W	10000	43	4.3
		Reconv.	9	36.7	89	4.68	8200	157000	Ret	406	0	0
									R	1412	27	38.2
									W	10000	18	1.8
		Reconv.	16	36.4	87	4.84	8000	88000	Ret	360	1	2.7
									R	508	12	47.2
									W	10000	22	2.2

1.94‰ in leucopenia. In the defervescent stage the mitosis index showed a lower level than the normal (2.3‰) in the former, because the nucleated

XV
the Mitosis in Leucocytosis

Appearance of mitosis																
Erythropoiesis							Leucopoiesis									
Baso.	Poly chr.	Oxy.	Pro.	Meta.	Ana.	Telo.	Mbl.	Unm. M.	Halfm. M.	Mat. M.	Meta M.	Eos.	Pro.	Meta.	Ana.	Telo.
10	26	6	17	12	6	7	2	4	10	8	0	2	16	6	4	0
6	11	4	10	4	3	4	0	6	10	10	0	6	20	6	0	6
7	20	19	18	12	6	10	0	6	12	8	0	8	16	8	6	4
5	9	3	7	6	2	2	0	2	6	8	0	2	10	4	0	4
12	23	8	15	10	4	14	2	4	8	4	0	6	12	6	4	2
8	16	9	14	8	5	6	2	4	10	10	0	8	20	8	6	0
2	13	18	20	3	3	7	1	5	10	11	0	0	13	6	5	3
2	12	5	8	6	2	. 3	0	2	8	8	0	0	8	4	4	2
11	29	17	20	6	11	20	1	3	13	5	0	3	13	8	2	2
6	23	13	9	6	9	18	4	4	12	20	0	0	22	8	6	4
10	26	9	20	10	4	11	2	2	14	8	0	2	16	4	6	2
4	19	7	11	6	4	9	2	4	8	4	0	4	12	4	2	4
4	18	8	12	5	3	10	0	8	12	24	0	0	28	4	8	4
4	14	9	13	3	3	8	0	4	6	8	0	0	8	4	4	2
3	6	3	4	3	3	2	0	4	10	8	0	0	12	4	2	4

cells had increased abundantly. In the same way, a higher level than the normal (2.52‰) was found in the latter, because they had decreased strik-

TABLE
Frequency of Every Phase

Name, Age, Sex	Disease	Stage	Day of the illness	Temp °C	Hgb. %	R.B.C. in B. (Million)	W.B.C. in B.	Nucleated cells in B.M.	Counted cells		M. counts	M. I. %
S.S. 27 ♀	Para-typhoid A	Acme	6	38.9	74	3.12	4000	112000	Ret.	674	1	1.5
									R.	536	13	48.5
									W.	10000	18	1.8
		Fever-abating	27	37.1	78	3.68	5400	105000	Ret.	362	0	0
									R.	1072	19	35.5
									W.	10000	17	1.7
		Reconv.	41	36.7	70	3.66	6200	95000	Ret.	642	0	0
									R.	1902	38	40.0
									W.	10000	23	2.3
M.M. 25 ♀	Para-typhoid A	Acme	12	38.8	73	3.70	5200	180000	Ret.	1592	3	1.9
									R.	1956	37	37.9
									W.	10000	20	20.0
		Fever-abating	26	38.2	79	3.89	5200	135000	Ret.	988	2	2.0
									R.	1942	49	50.5
									W.	10000	38	3.8
		Reconv.	33	36.3	81	4.14	5600	144000	Ret.	652	0	0
									R.	2682	62	45.4
									W.	10000	21	2.1
H.Y. 25 ♂	Typhoid	Fever-abating	14	37.4	84	4.48	6300	87700	Ret.	518	0	0
									R.	1040	20	38.5
									W.	10000	21	2.1
		Reconv.	21	36.7	80	4.42	5800	78300	Ret.	820	2	2.4
									R.	1406	33	46.9
									W.	10000	23	2.3
		Reconv.	28	36.3	86	4.72	6800	94500	Ret.	860	2	2.3
									R.	1866	36	38.6
									W.	10000	24	2.4
H.K. 33 ♀	Typhoid	Acme	31	39.1	79	4.15	7000	106400	Ret.	402	0	0
									R.	982	23	46.8
									W.	10000	24	2.4
		Fever-abating	63	38.3	77	4.22	4800	136500	Ret.	1396	4	2.9
									R.	2938	72	49.0
									W.	10000	28	2.8
		Reconv.	83	36.6	84	4.40	3300	157500	Ret.	662	1	1.5
									R.	1691	52	58.1
									W.	10000	22	2.2
S.T. 22 ♀	Typhoid	Acme	28	39.1	62	3.16	2600	78400	Ret.	1080	2	2.0
									R.	3020	128	42.4
									W.	10000	20	2.0
		Fever-abating	52	36.3	61	2.98	2500	69800	Ret.	78	0	0
									R.	3086	62	40.2
									W.	10000	22	2.2
		Acme	60	39.4	54	2.64	3200	87450	Ret.	210	0	0
									R.	2390	43	35.9
									W.	10000	16	1.6

ingly, attaining the normal level in the reconvalescence. Between leucocytosis and leucopenia there is a great difference in the mitotic function;

XVI
of the Mitosis in Leucopenia

Appearance of mitosis																
Erythropoiesis							Leucopoiesis									
Baso.	Poly chr.	Oxy.	Pro.	Meta.	Ana.	Telo.	Mbl.	unm. M.	Halfm. M.	Mat. M.	Meta M.	Eos.	Pro.	Meta.	Ana.	Telo.
2	4	7	8	2	1	2	0	2	8	8	0	0	4	8	4	2
2	7	10	6	2	2	9	0	2	6	4	0	4	6	2	4	4
8	16	14	16	8	4	10	0	4	10	8	0	0	14	6	2	0
14	12	11	10	11	5	11	2	4	8	6	0	0	6	8	4	2
15	24	10	29	8	3	9	4	4	14	16	0	0	14	16	4	4
11	36	15	28	10	12	12	0	2	12	6	0	0	12	4	4	0
5	7	8	11	7	0	2	0	4	8	8	0	0	8	6	2	4
4	17	12	13	7	2	11	2	6	8	6	0	2	14	6	4	0
10	14	12	19	8	4	5	0	9	10	6	0	0	18	4	0	2
4	8	11	5	9	6	3	0	0	8	12	0	4	2	12	10	0
17	28	27	26	25	12	9	2	8	10	8	0	0	4	16	8	0
13	24	15	22	14	10	6	0	6	4	10	0	2	8	6	6	2
39	61	28	56	24	13	35	1	7	5	7	0	0	8	8	3	1
15	22	17	27	14	11	10	0	2	12	8	0	0	16	2	2	2
7	14	22	17	9	7	10	2	4	6	4	0	0	2	6	6	2

in leucocytosis it is frankly accelerated while in leucopenia it is equally frankly lowered, attaining the normal level after the reconvalescence, but

there is not much difference in the maturation, the outflow into the circulating blood and the destruction of the white blood cells. The mitotic function was related intimately with the severity and the duration of the illness, and it could not be observed that the demand and the destruction of the white blood cells in the periphery were accelerated especially in the case of leucopenia in comparison with those in the case of leucocytosis.

The frequency of the 4 phases of mitosis observed in normal person was highest in prophase, followed by metaphase, anaphase and telophase in the order named. The ratio of the frequency of the 4 phases is proportionate to the ratio of their duration, and this fact applied to the cases both of leucocytosis and leucopenia.

Conclusions

The authors have observed 5 cases of leucocytosis (4 cases of Japanese encephalitis and 1 of scarlet fever) and 5 cases of leucopenia (3 cases of typhoid fever and 2 of paratyphoid A) and studied the mitotic patterns of the nucleated cells in the bone marrows, obtained by serial puncture during the various stages of the disease, using 5 normal persons as controls.

1. In normal persons, the mitosis index of the nucleated cells in the bone marrows amounted to 41.8‰ in the erythropoietic system and to 2.4‰ in the leucopoietic system. Among the erythropoietic system, the basophil erythroblasts showed the highest mitosis index and among the leucopoietic system, the half-matured myelocytes, but in the actual percentage of mitosis the polychromatic erythroblasts and the half-matured myelocytes occupied the highest position in the two systems. In the reticuloendothelial system mitosis was seen chiefly in the plasma cells, in which the mitosis index was about 1‰.

2. A rise in mitosis of the erythropoietic system was observed in the case of both leucocytosis and leucopenia when the disease took a prolonged course and a peripheral anemia was incurred.

3. In the cases of leucocytosis the mitosis index of the leucopoietic system in the bone marrow rose remarkably in the peak stage, dropped below normal in the defervescence, but recovered the normal value in the convalescence.

4. In the cases of leucopenia the mitosis index of the leucopoietic system was observed to remain on a perceptibly subnormal level except in the defervescent stage when it transiently showed a value somewhat higher than the normal, but returned to the normal in the stage of convalescence.

5. In leucocytosis mitosis of the leucopoietic system centered upon the matured myelocytes in the peak stage, on the half-matured myelocytes in the defervescent and in the convalescent stages, mitosis first leaning over to the half-matured and unmatured myelocytes and finally over to the

half-matured myelocytes, as in normal persons. In the leucopoietic system of leucopenia a drop of mitosis appeared remarkably in the half-matured and the matured myelocytes in the peak stage. The drop shifted gradually more to the matured myelocytes in the fever-abating stage, returning then gradually to the normal. Mitosis recovered from the left to the right in the bone marrow.

6. In the reticuloendothelial system the mitosis index showed an increase in the peak stage of leucocytosis.

7. If the leucocyte level of the peripheral blood can be divided into the three groups of less than 6,000 (subnormal), 6001–9,000, (normal), and more than 9,001 (supernormal), the mean mitosis index in the leucopoietic system stands at 2.17‰ in subnormal, at 2.5‰ in normal and at 3.2% in supernormal cases. A rise or drop in mitosis in the bone marrow is followed by a similar change in the peripheral blood about 4–7 days afterwards.

8. Both in leucocytosis and leucopenia of infectious diseases the leucocyte count in the peripheral blood is parallel to the mitosis index in the bone marrow. Therefore the nucleated cells in the bone marrow increased frankly in leucocytosis, while they decreased equally frankly in leucopenia, and the nucleated cell count in the former is not rarely twice or three times as much as that in the latter.

9. The genetic cause of leucopenia cannot be simply assumed to lie in an insufficiency of the bone marrow. Both in leucocytosis and leucopenia there is not much difference in the maturation, the outflow into the circulating blood and the destruction of the white blood cells, but a perceptible inequality is observed in their mitotic function; in leucocytosis it is frankly accentuated, while in leucopenia it is equally frankly lowered. As the disease improves, the mitotic function gradually becomes lower in the former, but accelerated in the latter, attaining the normal level in convalescence.

10. The frequency of the four phases of mitosis observed was highest in prophase, followed by metaphase, anaphase and telophase in the order named.

References

1) Saitô Ono, Tohoku J. Exp. Med., 1958, **67**, 253.
2) Saito Shimoyama, *ibid.*, 1958, **67**, 265.
3) Ernst, Handb, Allg. Path., 1915, 3 Bd, 372.
4) Lindenbaum, Fol. Haemat., 1930, **39**, 501.
5) Segerdahl, *ibid.*, 1934, **52**, 68.
6) Picena, Rev. Med. Rosario, 1937, **27**, 1167–1354.
7) Osgood, J. Amer. Med. Assoc., 1937, **109**, 933.
8) Müllendorf, Schweiz. Med. Wschr., 1938, 119.

9) Henning, Erg. Inn. Med., 1939, **56**, 372.
10) Rohr, Das menschliche Knochenmark, Georg Thieme, Leipzig, 1940, p. 70.
11) Fiesch, Haematologica, 1938, **19**, 539.
12) Miyamoto, Acta Haematol. Jap. (Jap.), 1955, **18**, 647.
13) Miyamoto & Saito, *ibid.*, 1955, **18**, 246.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

A Few Improvements on the Technique of Skin Transplantation in Mice

By

Sakari Sato, Shingo Kakizaki, Koichi Mizutani,
(佐藤 澂) (柿崎真吾) (水谷恒一)
Keizo Miura, and **Yoshinobu Ishikawa**
(三浦慶造) (石川義信)

From the Surgical Clinic of Prof. S-T Katsura, Tohoku University Hospital, Sendai

(Received for publication, January 22, 1958)

The occasions calling for homotransplantation of skin in animals are recently growing more and more frequent, in the scientific fields, especially, in biology, genetics and medicine.

In the domein of medicine, this process has been taken up as a step in the study of homotransplantation of organs as well. Skin grafting is easier in technical execution than other organ or tissue transplantation, especially in orthotopic grafting. The advantage of the method is easy to observe the subsequent development of the treated site and the functions of the graft. For example, hair growth and pigmentation can be readily examined following skin grafting.

Since Billingham and Medawar[1] have described their transplantation technique in detail, improvements (chiefly on the covering materials) have been reported on a few points,[2),3),4)] but no essential change has been broght about in the technique. The authors have been achieving a series of improvements and simplifications in the technique during experimental studies for some years.

A curt summary of these achievements have been previously reported[5)], and in this paper, the techniques of skin transplantation of which we are currently adopting, including the improvements achieved after the previous report, are described in detail.

There are several methods in skin grafting, namely, of using either pedicle or free graft, of grafting the skin in any thickness from the whole skin including all the layers down to the epidermis only, of covering the bed to be grafted with one or more than one pieces of transplanted skin and of covering up the whole bed by closely fitting the edges of the skin of the grafted site of the recipient and the skin of the graft (fitted style) or leaving a certain part of the bed uncovered (open style).

In this report, we will limit ourselves to the transplantation of free whole thickness grafts in fitted style, in respect to the grafting method.

Anaesthesia

In the earlier stage of our study, we worked with mice, generally, anaesthetized with intra-abdominal injection of 0.15 cc. per 10 gram of body weight of nembutal solution of 3 mg. in 1 cc. in saline, but at present we are operating without any anaesthesia. No inconvenience has been encountered.

Fixing

Rubber loops are fixed on the four corners of wood plates of about 12 cm. × 13 cm. and an aluminium washing clip is tied to one of the end of the loop. As the rubber loops maintain adequate elasticity, they do not lose their hold by motion of the animal's limbs and such binding does not subject the animals to overstrain (Fig. 1).

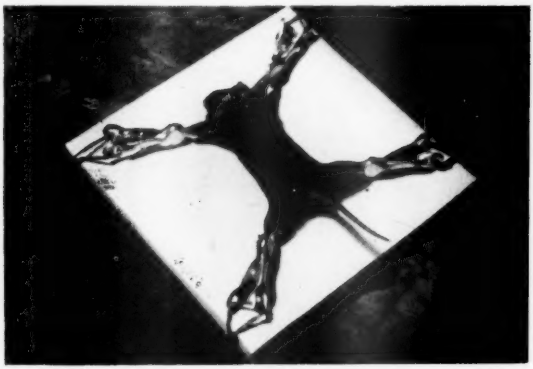


Fig. 1

Preparation of the Graft

Usually, the grafts are cut out from the skin of the back and the chest of the animal. In the phase of proliferating hair-follicles, however, the skin grows too thick for any transplantation, and such skin specimens must be avoided for experiments. In mice of colored breed, pigments are deposited therein, so that the thickening is seen at a glance, but in white species of mice, we have to pinch up the skin to feel its thickness. In such cases, the blood vessels cannot be visualized due to the thickening of the skin.

At the outset, the hair must be removed, but as shaving causes injury to the skin, we use depilatory cream instead of a razor and the completely beared site is cleaned with humid gauze till scarcely any odor of the cream

is left. No harmful effect due to the depilatory cream has ever been experienced. Two per cent alcohol solution of mercurochrome is used for disinfection and then the animal is covered under a sheet of vinyl plastic previously soaked in positive ion soap solution and with a hole of suitable area cut at its center. The skin is pinched up with a midget hook-forceps and the specimen of the required area is cut with a fine pair of curved scissors at one stroke.

The epidermis side of the specimen is then stuck on the finger-tip and the panniculus carnosus and the panniculus adiposus must be dissected with the same pair of scissors. If the piece is transplanted without removing these layers, they would act detrimentally except in auto-transplantation, so that the chance of successful results is extremely minimized. The grafts thus prepared are preserved in physiological saline till the bed of transplantation is completed.

Preparation of the Transplantation Bed

The processes of depilation and disinfection are the same as in the preceding. The skin of the site to be grafted is picked up with a midget hook-forceps and a very shallow V-shaped cut is made on it with a pair of fine curved scissors. When the cut goes too deep, the bed may be injured, so that it is better to cut it as shallow as possible. First, pick up the apex of the V-shaped cut with the forceps which is held with the left fore-finger and thumb, then the smaller end of the ablator devised by us (Fig. 2) is inserted between the skin and the muscle layer, using the left middle finger as support. When the ablation has gone to some extent, the other end of the ablator is used to peel the skin further, while the ablation is helped on with a pair of fine curved scissors, now and then releasing the pincers hold to control the form of the line of ablation (Fig. 3). Thus, a bed of any shape and area may be formed at will. In the processes of the operation, proper care is required to avoid injury the muscle layer, because the result is worse when the layer is injured or a blood vessel running on its surface is broken.

Grafting

The authors have previously sutured the edges with blood vessel threads, but at present we can dispense with sutures by adopting the following procedure. First, absorb the surplus humidity on the surface of the bed with dry aseptic gauze and *leave it standing at room temperature for* 10 *to* 15 *min.* The bed will adequately dry up and dewy lymph will ooze on the raw surface (Fig. 4). Then, stick the graft prepared previously on the operator's finger-tip with the outer skin surface down and trim it into a similar shape but about 10% larger in dimension than the bed by clipping

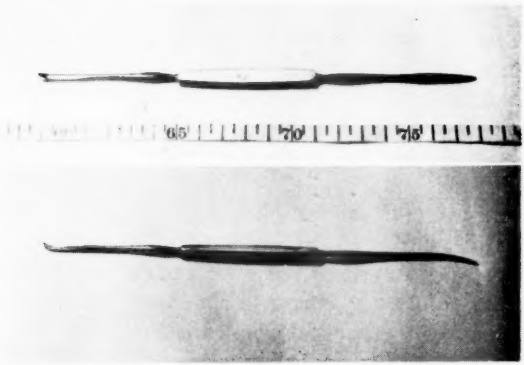


Fig. 2

Fig. 3

off the superfluous parts. Press the graft on the finger-tip upon the bed and it will become fixed on it, the edges being adapted. A few minutes after it is transplanted, it becomes firmly adherent to the bed and will not be abraded easily by external forces (Fig. 5).

Covering and Dressing

Most of the researchers would cover the grafted sites with plastic sheets or fabrics, knit of special fibres, and place elastic adhesive tapes over it to hold them in place. In the earlier stage of our study, we also used to cover the site with polyvinyl or cellophane film preserved in 70% alcohol, sealed the edges of the film to the skin of the host animal with collodium, then placed a small cotton ball as pressor and wrapped the site including the chest and upper abdomen with adhesive tapes. Such a method, how-

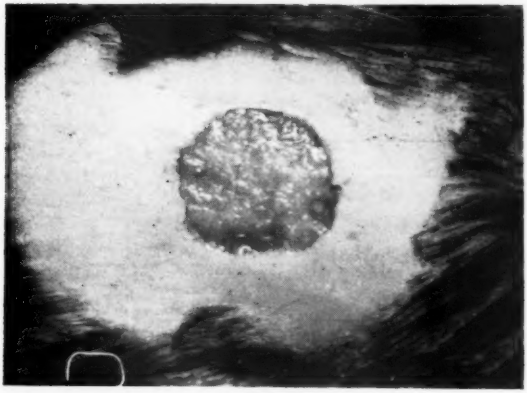


Fig. 4

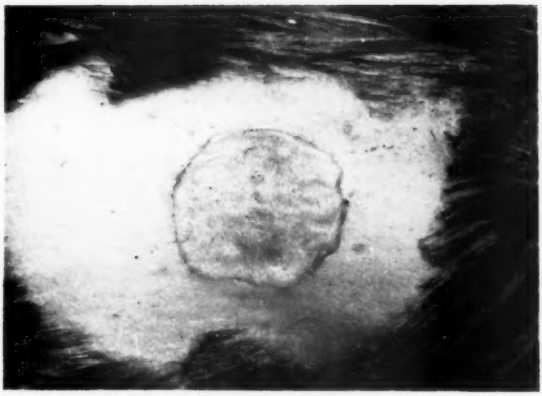


Fig. 5

ever, has the following disadvantages: 1) Before removal of the dressing, we could not only observe the grafts ocularly, but also palpate its hardness for examination. Even when the cotton ball is dispensed with and transparent cellophane tapes were used, we could not palpate it. (For evaluating the degree of vital adhesion, palpation is very serviceable besides ocular examination.) 2) The mice feel unpleasant under the dressing and keep twitching the skin, so that the operated site is hard to keep at rest. 3) Sometimes the inside of sheet becomes humid and multiplies the chance of infection. 4) When removing the dressing 7 to 10 days after the operation, sometimes the sheet is found adhered to the graft and the transplantation is impaired. Particularly, when sutures are not applied, such dressings are nothing but harmful, so we are keeping the animals in a glass

jar as shown in Fig. 6 without any dressing, but we have had no experience of failure by infection. Dislocation by mechanic stimulus which was very rare, was mostly due to the effort of the animal itself by biting the applied graft. This accident had been effectively suppressed by smearing saturated picric acid around the grafted area.

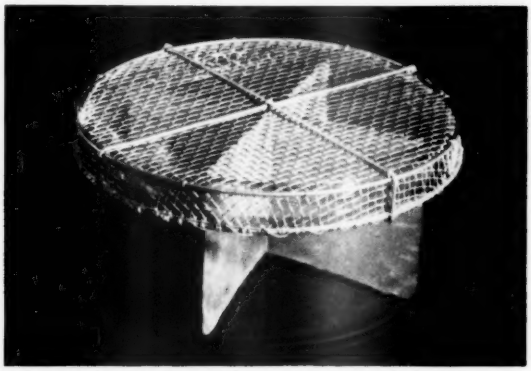


Fig. 6. With tin-plate partitions.

Conclusion

The authors have described the technique currently employed in experimental transplantation of free skin in its whole thickness in mice, including the improvements achieved during the past few years of experimental study. The method described herein has the following merits:

1. It is simple in operation.
2. The transplantation is highly successful, without failures due to peeling and infection.
3. Ocular examination and palpation are not hampered from the earliest stage of the transplantation.

References

1) Billingham & Medawar, J. Exp. Biol., 1951, **28**, 385.
2) Bryant & Bernard, Transplantation Bulletin, 1955, **2**, 133.
3) Silmer, Bond & Eichwald, Transplantation Bulletin, 1955, **2**, 38.
4) Hardin, Transplantation Bulletin, 1954, **1**, 186.
5) Ishikawa, Sato & Abo, Transplantation Bulletin, 1957, **4**, 108.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Studies on Cirrhosis of the Liver

IV. The Relations between the Mortality from Liver Cirrhosis and the Mortality from Alcoholism, and between the Mortality from Liver Cirrhosis and Alcoholic Consumption

By

Takashi Nakamura, Shozo Nakamura, Etsuko Kawamura,
(中 村 隆) (中 村 省 三) (河 村 悦 子)
Taketsugu Kaneko, Kanae Sugawara, Yoshiaki Katakura,
(金 子 長 次) (菅 原 香 苗) (片 倉 吉 昭)
Tsuneo Isono and **Toshimi Suzuki**
(磯 野 恒 雄) (鈴 木 敏 巳)

From the Medical Clinic of Prof. T. Nakamura,
Tohoku University School of Medicine, Sendai

(Received for publication, January 23, 1958)

In previous reports[1),2)], the close relationship between alcoholic intake and the production of liver cirrhosis was pointed out from the results of liver biopsy and liver function tests in alcoholics. For the purpose of further investigation of this problem statistical studies on the relation between the mortality from liver cirrhosis and that from alcoholism or alcoholic consumption are attempted.

The relation between the mortality from liver cirrhosis[3)] and that from acute and chronic alcoholism[3)] in the countries of the world (Chile, Portugal, France, Italy, Switzerland, U. S. A., Japan, Union of South Africa, Denmark, Australia, Canada, Ceylon, Scotland, Sweden, Israel, New Zealand, Holland, Norway, England and Wales, Northern Ireland, Ireland, Finland, and Egypt) is illustrated in Fig. 1. The mortality from liver cirrhosis is for the year 1950, and that from alcoholism for the year 1949. When the mortality for these years is missed, that for the nearest year is used. The correlation coefficient is $+0.90$, and statistically significant at the level of less than 0.1%.

The relation between the mortality from liver cirrhosis[1)] and alcoholic consumption per capita in the countries of the world (France, Portugal, Italy, Austria, Switzerland, U.S.A., West German Republic, Japan, Union of South Africa, Canada, Australia, Sweden, Holland, Israel, Norway, Finland, New Zealand, Ireland, Denmark, and Egypt) is illustrated in Fig. 2.

It is difficult to know the exact amount of alcoholic consumption in the countries of the world. In the present investigation alcoholic consump-

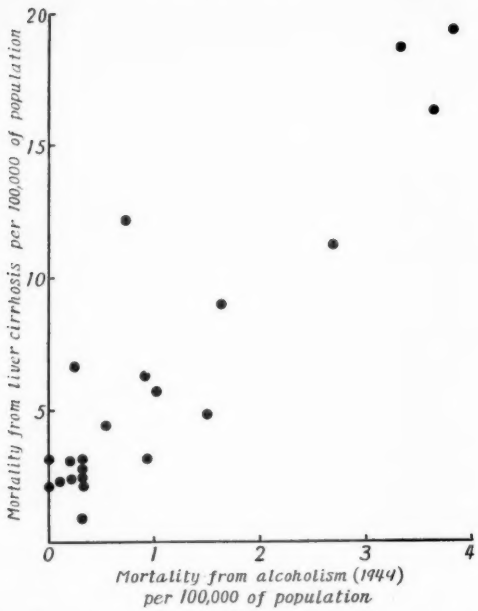


Fig. 1. The relation between the mortality from liver cirrhosis and the mortality from alcoholism in the countries of the world.

tion is calculated from output, exports, and imports of wine and output of beer[5),6)], and consumption of distilled spirits is not included in the calculation except in Japan and U.S.A., in which alcoholic consumption including distilled spirits is obtainable[7),8)]. Although the calculated alcoholic consumption is not exact, it seems to be not very different from real consumption, since the amount of the consumption of distilled spirits is small as compared with that of wine and beer. It is supported by the fact that there is no marked difference between the calculated alcoholic consumption in the present investigation and that cited by Malignac and Colin[9)].

It is well known that in the United States of America both the annual mortality from liver cirrhosis and that from alcoholism decreased during the period of prohibition law[10)]. The present observations, showing positive significant correlations between the mortality from liver cirrhosis and that from alcoholism or alcoholic consumption in the countries of the world, suggest that the mortality from liver cirrhosis in the countries of the world depends on the degree of alcoholic intake of the populations.

In the prefectures of Japan, on the other hand, there are no significant

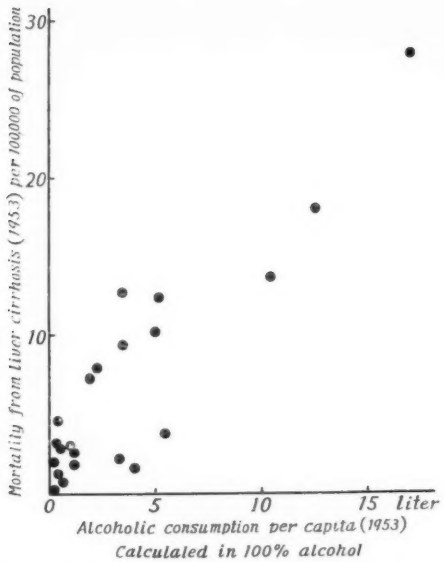


Fig. 2. The relation between the mortality from liver cirrhosis and the alcoholic consumption in the countries of the world.

correlations between the mortality from liver cirrhosis (1937)[11),12)] and that from alcoholism (1937)[11),12)], and between the mortality from liver cirrhosis (1954)[13)] and alcoholic consumption (1955)[7)] (Figs. 3 and 4). It may be due to the fact that the variations of alcoholic consumption and the mortality from alcoholism in the prefectures of Japan are slight as compared with those in the countries of the world.

In previous reports[14),15)], it was shown that there was positive significant correlation between the mortality from liver cirrhosis and mean annual temperature in the countries of temperature below 16°C whereas there was no significant correlation in the countries of mean annual temperature over 16°C. The investigation of the relation between alcoholic consumption[5),6),7),8)] and mean annual temperature[16)] in the countries of the world reveals that there is also positive significant correlation between them in the countries of mean annual temperature below 16°C and no significant correlation in the countries of mean annual temperature over 16°C.

The relations between mean annual temperature, alcoholic consumption and the mortality from liver cirrhosis in the countries of the world are as illustrated in Fig. 5. It may be not unreasonable to infer that at first alcoholic consumption is influenced by temperature and the mortality from liver cirrhosis is secondarily influenced by temperature.

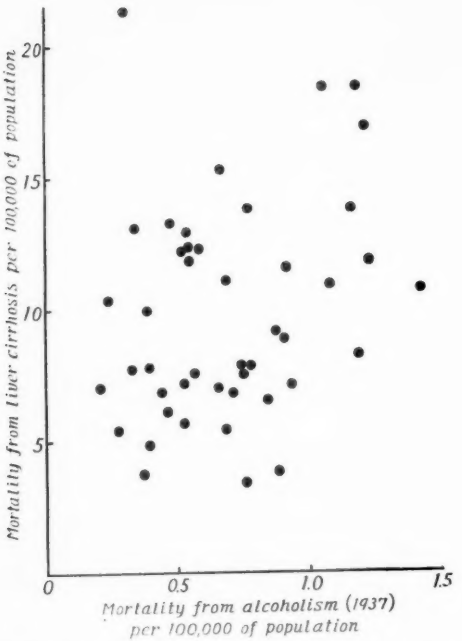


Fig. 3. The relation between the mortality from liver cirrhosis and the mortality from alcoholism in males in the prefectures of Japan.

Although the previous papers[11),15)] showed significant correlation between the mortality from liver cirrhosis and mean annual temperature in the prefectures of Japan, no significant correlations are recognized in the present observations between alcoholic consumption and mean annual temperature and between the mortality from liver cirrhosis and alcoholic consumption. Hence, in the prefectures of Japan the relation between the mortality from liver cirrhosis and temperature can, contradictionary to that in the countries of the world, not be explained on the view of alcoholic consumption. Further investigation is required in this respect.

The correlation between the mortality from liver cirrhosis and alcoholic consumption in the countries of the world suggests that alcoholic consumption may have influence upon dietary intake and the mortality from liver cirrhosis is secondarily influenced by the latter. However, no significant correlations are observed between the mortality from liver cirrhosis and intake of protein, fat, and carbohydrate or the ratio of calories of these essential nutrients to total calories.

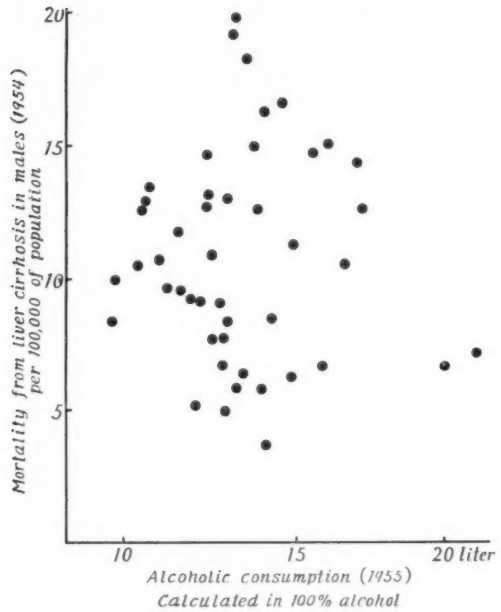


Fig. 4. The relation between the mortality from liver cirrhosis and the alcoholic consumption in the prefectures of Japan.

Summary

1. There are significant correlations between the mortality from liver cirrhosis and that from alcoholism and between the mortality from liver cirrhosis and alcoholic consumption per capita in the countries of the world. The mortality from liver cirrhosis in the countries of the world seems to depend on the degree of alcoholic intake of the populations.

2. There is also significant correlation between alcoholic consumption and mean annual temperature in the countries of mean annual temperature below 16°C, whereas there is no significant correlation in the countries of temperature over 16°C.

3. In the prefectures of Japan, the correlations between the mortality from liver cirrhosis and that from alcoholism, between the mortality from liver cirrhosis and alcoholic consumption, and between alcoholic consumption and mean annual temperature are not significant statistically.

References

1) Nakamura, T., Nakamura, S., Sugawara, Katakura, Isono, Suzuki, Kaneko & Taki-

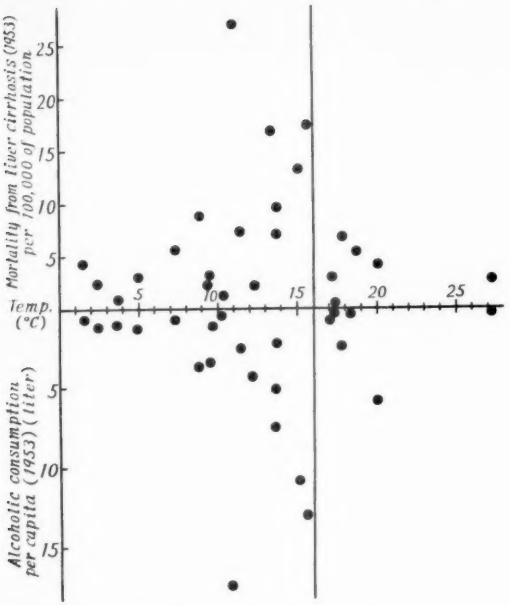


Fig. 5. The relations between the mean annual temperature and the mortality from liver cirrhosis or the alcoholic consumption in the countries of the world.

zawa, Nihon Naikagakkai Zasshi (Jap.), 1957, **46**, 993.

2) *Idem*, Tohoku J. Exp. Med., in press.

3) World Health Organization of the United Nations, Epidemiological & Vital Statistics Report, 1950, **3**, 275 ; 1951, **4**, 197.

4) *Idem*, *ibid.* 1956, **9**, 174.

5) Statistical Office of the United Nations, Statistical Yearbook, 1954, **6**, 177.

6) Food & Agriculture Organisation of the United Nations, Yearbook of Food & Agriculture, 1954, **8**, (2), 195.

7) Tax Administration Agency of Japan, Table on Alcoholic Consumption for the Year 1955 (Jap.), 1956.

8) Statistical Report Division, U.S. Department of Commerce, Statistical Abstracts of the United States, 1956, **77**, 814.

9) Malignac & Colin, l'Alcoolisme, Collection Que Sais-Je, translated into Japanese by Miura & Horiuchi, Hakusui-sha Tokyo, 1956, p. 58.

10) Rowntree, J. Am. Med. Ass., 1927, **89**, 1950.

11) Statistics Bureau of the Japan Cabinet, Vital Statistics (Jap.), 1937, p. 2.

12) *Idem*, Mortality Statistics (Jap.), 1937, p. 2.

13) Statistics & Survey Division of the Japan Ministry of Health & Welfare, Vital Statistics (Jap.), 1954, **1**, 146 ; 1954, **2**, 246.

14) Nakamura, T., Nakamura, S., Sugawara, Katakura, Isono & Kawamura, Nihon

Naikagakkai Zasshi (Jap.) 1956, **45**, 858.
15) *Idem*, Tohoku J. Exp. Med., 1957, **65**, 255.
16) Tokyo Astronomical Observatory, Rika Nempyo (Jap.), 1956, **29**, Meterol., p. 7 & 78.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Production and Purification of Receptor Destroying Enzyme (RDE)

(Studies on several inhibitors againts influenza viruses, 1st Report)

Keiji Kawamura
(川村 慶二)

The Department of Bacteriology, School of Medicine, Tohoku University, Sendai; Director: Prof. M. Kuroya

(Received for publication, March 13, 1958)

In order to obtain a potent RDE preparation, biological studies on the 4Z strain of V. cholerae and on production media and chemical studies on purification procedures have been conducted. The conclusions may be summarized as follows:

1) The original 4Z strain was plated out on nutrient agar and colony selections were performed. The characteristic S from colony produced more a potent RDE than the RS or R type colony. Moreover, a constant titer of RDE was always obtainable with the former substrain.

2) In order to find the best production medium, 1% nutrient agar, 1% peptone water, meat extract peptone broth and meat infusion broth were examined. The former two were more favourable for this purpose, with peptone water being more profitable for further purification procedures.

3) Different kinds of peptone were examined for the production of RDE in peptone water with Neo-peptone and Teruuchi-peptone, showing the highest titer.

4) The addition of hog stomach mucin to peptone water increased the production of RDE as much as 20 times, when compared to plain peptone water. The optimum concentration of mucin was 15–25 mg/ml., and in this medium a maximum titer was obtainable after 16–18 hrs. incubation.

5) Instead of mucin, egg-white may also be used to stimulate the production of RDE.

6) Purification procedure from the culture filtrate was examined, with adsorption to and elution from chicken red blood cells being the most important step for purification.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Studies on Influence of Protein on the Liver Injury due to Chloroform and Carbon Tetrachloride

By

Takashi Nakamura, Shozo Nakamura and **Etsuko Kawamura**
(中 村 隆) (中 村 省 三) (河 村 悦 子)

From the Medical Clinic of Prof. T. Nakamura,
Tohoku University School of Medicine, Sendai

(Received for publication, January 23, 1958)

There are many papers which reported the protective effects of nutrition against liver injury due to poisons. Goldschmidt[1] observed that a high protein diet, previous to the anesthesia with chloroform, markedly reduced the incidence of hepatocellular necrosis in rats. Miller[2] reported that chloroform anesthesia could be tolerated with little evidence of liver injury in well-fed healthy dogs. Hove[3] showed higher survival rate in the rats on high protein diet than on low protein diet in the experiment of carbon tetrachloride poisoning. All these reports stressed the protective effects of high protein diet, which is one of the basis for the high protein therapy for liver diseases.

On the other hand, papers against these results have been frequently published. In the experiment of Drill *et al.*[4], bromsulphalein retention test showed a greater degree of hepatic dysfunction in the dogs fed with a normal protein diet than those receiving a low protein high carbohydrate diet, and hepatocellular necrosis was not observed in the dogs fed with the low protein diet but was present in 3 of 6 dogs fed with the normal protein diet. According to Campbell and Kosterlitz[5], the rats placed on a protein-free diet for 3 days showed the least liver damage, whereas those on a 18% casein diet showed the highest incidence of hydropic degeneration.

Thus the results are not always in accordance with each other. In the experiment of long term poisoning of carbon tetrachloride of the authors, fibrosis of the liver of rats was more extensive on a high protein diet than on a low protein diet[6]-[8]. What caused such disaccordance of the results in connection with the influence of protein on liver injury? Is it due to the difference of poisons? Is it due to the difference of judging the results on the basis of death rate of experimental animals, pathological changes of the liver or liver function tests? Or is it due to the assumption as proposed by Campbell and Kosterlitz[5] that low protein diet is effective

when the duration of protein deficiency is short whereas high protein diet is effective when the duration is long.

In order to answer these questions, rats were administered with chloroform and carbon tetrachloride after they were fed with low protein or high protein diet for various periods. Death rate and pathological findings of the liver and kidney were studied in these animals.

Experimental

Method

The composition of experimental diets employed is shown in Table I.

Table I

Composition of Experimental Diets

Figures express percentages.

Constituent	5% casein diet	30% casein diet	18% yeast diet
Yeast	0	0	18
Casein	5	30	0
Sucrose	85	60	72
Lard	5	5	5
Cod liver oil	1	1	1
Osborne-Mendel's salt mixture	4	4	4

Daily vitamin supplements: thiamin 50 μg., riboflavin 50 μg., pyridoxine 20 μg., Ca-pantothenate 100 μg., and nicotinamide 500 μg.

Rats were fed with 5% casein and 30% casein diet for 5 days, 2 weeks, 4 weeks and 8 weeks respectively, and thereafter administered with chloroform or carbon tetrachloride by stomach tube. Deaths were recorded every 12 hours. Three days after the administration of the poisons all surviving rats were killed in order to study the liver and kidney pathologically. Chloroform and carbon tetrachloride were administered at the level of 0.35–0.635 and 1.7–1.75 cc./kg. body weight respectively. Preliminary experiment established that this level of chloroform or carbon tetrachloride would kill about 50% of the rats on the high protein diet 3 days after the administration. However, comparison of death rate was occasionally unsatisfactory due to low mortality of rats. Chloroform was used as 20–40% solution of olive oil, while carbon tetrachloride was used alone.

Histologically the ratio of necrosis of liver cells to specimens was calculated. There may be a possibility that even under the same condition of sex of animals and amount of poison death rate and pathological changes of the liver may vary at different time of experiment. Therefore, comparisons were made only between two dietary groups which were fed

at the same time. In some experiments the difference of resistance to poison appeared to be influenced by body weight, and an additional experiment was undertaken to investigate this problem.

Results

Experiment of chloroform poisoning

The results are shown in Table II.

TABLE II

Influence of Protein on Liver Injury Due to Single Administration of Chloroform

Duration of feeding before administration of $CHCl_3$	Sex of rats	$CHCl_3$ administered, cc/kg.	Diet	No. of rats	Initial body weight, g.	Body weight at the time of administration of $CHCl_3$, g.	Daily food intake, g.	Death rate after administration, %						Per cent of rats showing liver necrosis				Per cent of rats showing proximal tubular necrosis of the kidney
								12 hours	24 "	36 "	48 "	60 "	72 "	0% < , ≦30% VII	30% < , <60% VII	60% <	Total	
5 days	F	0.635	5% casein	14	139.9	134.4	11.1	0	14	36*	36	43	57	21	0	0	21	29
			30% "	12	130.1	134.4	11.6	0	0	0*	17	58	58	17	17	0	33	50
2 weeks	F	0.635	5% "	11	146.2	141.6	11.2	0	36*	64*	73	73	73	36	9	9	55	18*
			30% "	12	145.6	164.2	11.4	0	0*	8*	33	92	92	50	17	0	67	67*
4 "	F	0.35	5% "	15	135.2	121.6	10.4	0	7	7	13	27	27	26	20	0	47*	27
			30% "	15	97.8	140.1	10.0	0	0	20	40	53	60	7	0	0	7*	60
8 "	F	0.35	5% "	12	145.1	132.2	11.1	0	0	0	0	0	8	42	25	0	67*	8
			30% "	14	83.3	140.1	11.0	0	0	0	0	0	0	0	0	0	0*	29

* The difference between 5% casein diet group and 30% casein diet group is statistically significant.

Death rate: In the experiments of 5 day feeding and 2 week feeding, death rate of 5% casein group was higher than that of 30% casein group 1 or 1.5 days after the administration. The difference was statistically significant ($P<0.05$). In the experiments of 4 week feeding and 8 week feeding, no significant difference was observed between both dietary groups.

Pathology of the liver: Macroscopically, the liver was generally swollen, soft and friable, and presented a mottled appearance with yellowish-gray necrotic areas and reddish-brown hemorrhagic areas. Histological studies revealed coagulation necrosis with hemorrhage and round cell infiltration in the central and middle part of hepatic lobules, which occasionally extended all over the hepatic lobules. Sometimes fatty metamor-

phosis of liver cells was noticed at the periphery of the hepatic lobules, and hydropic degeneration of liver cells and congestion of blood were more or less observed in all groups.

The difference of frequency of hepatocellular necrosis between 5% casein and 30% casein groups was statistically significant in the experiments of 4 week feeding and 8 week feeding ($P<0.05$), while there was no significant difference in the experiments of 5 day feeding and 2 week feeding.

Pathology of the kidney: The kidney was swollen, friable, tarnished, and reddish-gray in color. Histologically, the epithelium of renal tubules showed cloudy swelling and hydropic degeneration, including coagulation necrosis of the epithelium of convoluted tubules and hyaline and granular casts in the lumen of tubules. No changes was, however, noticed in Bowman's capsule and glomerulus. On the whole, the degree of injury of the kidney were slighter than that of the liver.

The frequency of coagulation necrosis of the epithelium of renal tubules was significantly lower in 5% casein group than in 30% casein group in the experiment of 2 week feeding, whereas no statistically significant difference was observed in the other experiments.

Thus, in the experiment of chloroform poisoning, liver injury was severer on the low protein diet than on the high protein diet when the duration of protein deficiency was long. The death rate failed to run parallel with the degree of liver and kidney injury.

Experiment of carbon tetrachloride poisoning

The results are shown in Table III.

Death rate: In the experiments of 5 day feeding and 2 week feeding both dietary groups showed low death rate without statistically significant difference. In the experiment of 4 week feeding the death rate of 5% casein group 1.5 or 3 days after the administration of carbon tetrachloride was lower than that of 30% casein group. The difference was statistically significant ($P<0.05$). In the experiment of 8 week feeding the death rate of 5% casein group 1 day after the administration was significantly higher than that of 30% casein group ($P<0.05$).

Pathology of the liver: The changes of the liver was, macrosocpically and histologically, almost the same as observed in chloroform poisoning. In the experiment of 4 week feeding, the frequency of liver necrosis was significantly lower in 5% casein group or 18% yeast group than in 30% casein group ($P<0.05$), whereas no significant difference was observed in the experiments of 5 days feeding, 2 week feeding, and 8 week feeding.

Pathology of the kidney: The kidney showed almost the same change as in the chloroform poisoning, but the degree of injury was slighter.

TABLE III

Influence of Protein on Liver Injury Due to Single Administration of Carbon Tetrachloride

Duration of feeding before administration of CCl_4	Sex of rats	CCl_4 administered, cc/kg.	Diet	No. of rats	Initial body weight, g.	Body weight at the time of administration of CCl_4, g.	Daily food intake, g.	Death rate after administration, %						Per cent of rats showing liver necrosis				Per cent of rats showing proximal tubular necrosis of the kidney
								12 hours	24 "	36 "	48 "	60 "	72 "	0% ≦ VII < 30%	30% ≦ VII < 60%	60% ≦	Total	
5 days	F	1.75	5% casein	13	140.8	138.1	11.1	0	0	0	0	0	0	8	8	0	15	0
			30% "	14	154.6	161.9	11.3	0	0	7	7	7	14	36	7	0	43	0
2 weeks	F	1.75	5% "	11	135.9	138.5	11.4	0	0	0	0	9	9	9	9	0	18	0
			30% "	10	147.3	168.4	11.9	0	0	0	0	0	0	40	0	0	40	0
			5% "	14	62.7	58.2	5.9	7	14	14*	14*	21*	21*	0	14	0	14*	7
4 "	M	1.7	30% "	15	63.0	155.3	10.9	7	7	80*	80*	87*	87*	20	60	13	93*	0
			18% yeast	15	64.7	88.3	9.1	13	13	20*	27*	27*	27*	7	20	7	33*	0
			5% casein	4	54.1	59.6	5.9	50	75	100	100	100	100	25	25	25	75	25
8 "	M	1.6	30% "	7	64.8	197.6	11.6	0	0	57	57	71	71	43	43	0	86	0
			18% yeast	6	67.5	122.3	10.2	50	50	50	50	50	50	0	17	33	50	0

* The difference between 30% casein diet group and 5% casein or 18% yeast diet group is statistically significant.

Coagulation necrosis of the epithelium of tubules was observed in only 2 animals of the 5% casein group in the experiments of 4 week feeding and 8 week feeding. The frequency of coagulation necrosis, hydropic degeneration, and cloudy swelling showed no significant difference between 2 dietary groups.

Thus the results of carbon tetrachloride poisoning was different from those of chloroform poisoning. In the experiment of 4 week feeding, liver injury was milder and death rate was lower on the low protein diet than on high protein diet. Such tendencies was, however, not observed in the experiment of 8 week feeding.

In the experiment, in which the influence of body weight on the liver injury due to carbon tetrachloride was investigated, the frequency of liver necrosis of the rats in the group with an average body weight of 58.6 g. was significantly higher than that in the group with an average body weight of 172.1 g. ($P=0.0042$) (Table IV).

DISCUSSION

In the experiments it was noticed that liver injury of the low protein

TABLE IV

Influence of Body Weight on Liver Injury Due to Single Administration of Carbon Tetrachloride

Duration of feeding before administration of CCl_4	Sex of rats	CCl_4 administered, cc/kg.	No. of rats	Initial body weight, g.	Body weight at the time of administration of CCl_4, g.	Diet	Daily food intake, g.	Death rate after administration, %							Per cent of rats showing liver necrosis			
								12 hours	24 "	36 "	48 "	60 "	72 "		0% < , ≦ 30%	30% < , ≦ 60%	60% <	Total
3 days	F	0.175	12	175.6	172.1	10% casein**	12.0	0	0	0	0	0	0		21	0	0	21*
			17	57.2	58.6	10% " **	8.0	0	0	6	12	12	12		54	18	6	76*

* The difference between large body weight group and small body weight group is statistically significant.

** The composition of the 10% casein diet is as follows: casein 10%, sucrose 80%, lard 5%, cod liver oil 1%, and Osborne-Mendel's salt mixture 4%. Vitamins are added to supply the following amounts daily: thiamin 50 μg., riboflavin 50 μg., pyridoxine 20 μg., Ca-pantothenate 100 μg. and nicotinamide 500 μg.

group which was administered with chloroform was severer than that of the high protein group. On the contrary, liver injury of the low protein group which was administered with carbon tetrachloride was slighter than that of the high protein group in the experiment of 4 week feeding. Accordingly, chloroform and carbon tetrachloride poisoning showed quite different results.

Since a remarkable difference of body weight was observed between the two dietary groups which received carbon tetrachloride in the experiments of 4 week feeding and 8 week feeding, there seems to be a possibility that such a difference may be attributed to lower sensitivity to carbon tetrachloride of low protein group with light body weight. However, the experiment, which was undertaken to investigate this problem, revealed that the liver injury of light body weight group was, on the contrary to the conjecture, severer than that of the heavy body weight group. Taking the above-mentioned results into consideration, the liver injury of the low protein group which received carbon tetrachloride would be severer than that of the high protein group even in the experiment of 8 week feeding, if the influence of body weight were excluded.

When the results of the present investigation are compared with the former reports, they accord with Miller's report that liver injury of low

protein diet group which was administered with chloroform was severer than that of high protein group when protein depletion was over a certain limit. In the experiment of chloroform poisoning the difference between both dietary groups was not noticed when protein deficiency was slight. This result does not support Campbell's assumption that low protein diet gives the higher protection against liver injury if given for short periods before the administration of poisons whereas prolonged protein deficiency renders the liver highly susceptible to injury.

In the experiment of carbon tetrachloride poisoning, liver injury of the low protein group was milder than that of the high protein group when it was fed for 4 weeks. This observation supports more Drill's report than Hove's, and is in accordance with the previous reports of the present authors on prolonged carbon tetrachloride poisoning[6].

On the basis of death rate, Hove[3] admitted the protective effect of high protein diet on carbon tetrachloride poisoning. However, the experiments indicate that death rate fails to accord with the patho-histological changes of liver cells. Judging results only on death rate may be inappropriate for the index for the estimation of the degree of liver injury.

According to Campbell and Kosterlitz[5], carbon tetrachloride liver injury of the rats which were fed with no protein for 3 days was milder than that of the rats fed with 18% or 54% casein diet. The present experiment showed that liver injury of the rats on low protein diet was milder than that on the high protein diet in the experiment of 4 week feeding, but not in the experiments of 5 day feeding and 2 week feeding. The difference may be attributed to the difference that 5% casein diet was used in the present experiment while no protein was included in Campbell's diet.

Thus the studies of the authors on the influence of protein on the liver injury due to poisons show that there is difference between chloroform administration and carbon tetrachloride administration, namely, if the protein depletion is over a certain limit, high protein diet is protective against the liver injury due to chloroform poisoning, while low protain diet is protective against the liver injury due to carbon tetrachloride. When the former reports are reviewed on the basis of this result, the disaccordance of the former reports can be comparatively well explained.

It is an interesting question why chloroform and carbon tetrachloride, chemically very related to each other, would cause such a difference. A further investigation is expected on this line.

Summary and Conclusions

1. Rats were fed with 5% and 30% casein diet for 5 days, 2 weeks, 4 weeks, and 8 weeks, and were given chloroform or carbon tetrachloride

by stomach tube. Three days after the administration, they were killed in order to study the liver and kidney pathologically. The difference due to nutrition was investigated.

2. In the experiment of chloroform poisoning, the incidence of liver necrosis in the rats which were fed with 5% casein diet for 4 or 8 weeks before the administration of chloroform was significantly higher than that in the rats which were fed with 30% casein diet. In the experiment of carbon tetrachloride poisoning, on the other hand, the incidence of liver necrosis of the rats which were fed with 5% casein diet for 4 weeks was significantly lower than that of the rats which were fed with 30% casein diet.

3. Kidney injury was generally milder than liver injury, and it was milder in the experiment of carbon tetrachloride poisoning than in that of chloroform poisoning. The incidence of necrosis of the epithelium of renal tubules was significantly lower in the rats which were fed with 5% casein diet for 2 weeks before the administration of carbon tetrachloride than in those which were fed with 30% casein diet.

4. Death rate after the administration of the poisons failed to run parallel with the degree of liver injury and was considered to be inappropriate for the indicator for the estimation of the degree of liver injury after the administration of poisons.

Death rate was also not proportional to the degree of kidney injury.

5. The disaccordance of the former reports regarding the influence of protein on liver injury due to poisons can be comparatively well understood by this experiment, if it is explained on the difference of used poisons.

References

1) Goldschmidt, Vars & Ravdin, J. Clin. Invest., 1939, **18**, 277.
2) Miller & Whipple, Am. J. Med. Sc., 1940, **199**, 204.
3) Hove, Arch. Bioch., 1947, **17**, 467.
4) Drill, Loomis & Belford, J. Indust. Hyg. Toxicol., 1947, **29**, 180.
5) Campbell & Kosterlitz, Brit. J. Exp. Path., 1948 **29**, 149.
6) Nakamura, T., Nakamura, S., Sugawara, Katakura, Isono & Kawamura, Nihon Naikagakkai Zasshi (Jap.), 1956, **45**, 60.
7) *Idem*, *ibid*, 1956, **45**, 858.
8) *Idem*, Tohoku J. Exp. Med., 1957, **65**, 255.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Studies of Color Blindness around the Spot of Mariotte by Electrical Stimulation

By

Kei Watanabe
(渡 部 敬)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, January 31, 1958)

Introduction

A lot of papers have been published about the color blindness around the spot of Mariotte[1)-2)] and about the variability in size of the blind spot[3)-4)].

In the preceding paper[5)] the color-blind zone around the spot of Mariotte was investigated with special reference to the intensity and the wave-length of the test light, to the size of the test patch used and the stage of dark adaptation.

All these investigations are, however, based on subjective sensations of color, and the data obtained are not always consistent with one another. It seemed, therefore, to be desirable to carry out a similar investigation by a more objective method.

As retinal color processes may be measured by Motokawa's method[6)] without resort to color sensations, an attempt was made in the present investigation to determine the color sensitivity around the blind spot by this method.

Experimental

Method

Colored lights used were spectral lights of 650, 580, 530 and 470 mμ from an equal-energy spectrum. The test patch used was 60′ in visual angle for red and green lights, and 20′ for yellow and blue lights. The distance between the patch and the subject's left eye was always 100 cm., the right eye being shielded.

In order to illuminate various points on the retina, a fixation point, a small red right, was moved on a vertical screen in the middle of which the test patch was fixed, and at each position the measurement of electrical excitability after an illumination was carried out.

Details of the procedure to determine the electrical excitability are

given in Motokawa's papers[6)-8)], so that they may be omitted here.

After a brief illumination of the dark-adapted eye, the electrical excitability of the retina generally increases, and the effect of light is expressed in term of ζ, that is, a percentage increase above the resting level: $=100\ (E-E_o)/E_o$, where E and E_o are electrical excitabilities with and without pre-illumination. When ζ-values are plotted against the time after termination of pre-illumination, a curve of electrical excitability is obtained. At the fovea the maximum of electrical excitability lies at, 1, 2, 1.5 and 3 sec. for red, green, yellow and blue pre-illuminating lights respectively. The duration of illumination was 2 sec., if not otherwise stated.

The so-called rod-process may be measured by Motokawa's method, too. According to Motokawa *et al.*[9)-11)] the curve of electrical excitability for rods reaches a maximum at 4.5 seconds from the onset of illumination. So using a white light of 16 lux. of 40 msec. in duration and various colored lights of the same duration, the rod-process was measured around the blind spot, because the excitation of rods was considered to be significant for the saturation of hue.

All experiments were carried out in a dark room after dark adaptation of 30 minites.

Results

1) *Changes of the excitability curve near the blind spot*

Excitability curves for red, green, yellow and blue lights were obtained at various points around the blind spot.

The origin of coordinates was taken at a certain point within the blind spot, namely at a distance of 15° 30′ from the fovea on the horizontal meridian.

The curves obtained on the foveal side of the origin are shown in Fig. 1. Those obtained on the peripheral side are represented in Fig. 2.

As can be seen from the curves for red light of Fig. 1, the height of the curve increases towards the fovea, and the maximum is displaced from 2 sec. to 1 sec.

This change of curve form corresponds to a psychological change from a colorless sensation to a red sensation. The curve at 4° from the origin shows a maximum at about 1.5 seconds. The subjective sensation at this point was yellow just as may be predicted from the crest time 1.5 sec. of the excitability curve obtained at this point.

On the peripheral side, the change is similar in character to that found on the foveal side, but the change is more gradual (see Fig. 2 Red); the curve form characteristic of red light is not obtained until 7° from the origin.

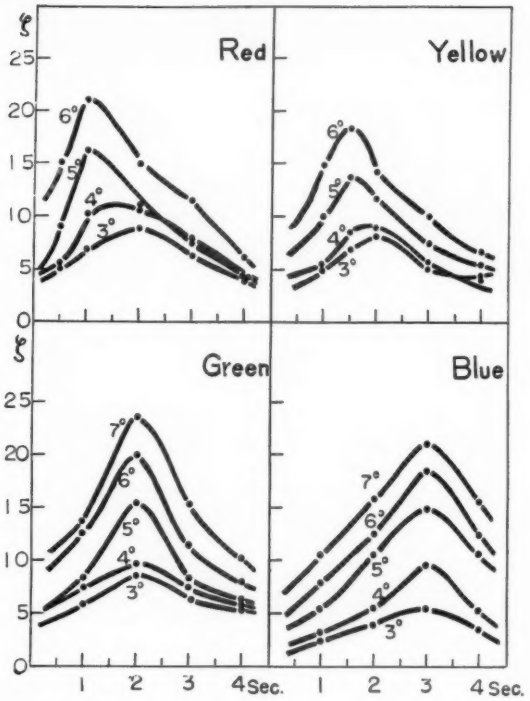


Fig. 1. Electrical excitability curves for colored lights at various points of horizontal meridian on foveal side of blind spot. Ordinates: ζ-values. Abscissae: time after end of pre-illumination. Number by each curve means distance from blind spot in visual angle.

The change of subjective sensation is also more gradual on the peripheral side; the red light appeared first colorless at just the margin of the blind spot. On the peripheral side of the spot it turned to yellow and then orange, and finally at 7° the proper red color appeared. This change of color sensation is accurately represented by the change of excitability curves. A similar correlation between excitability curves and the color sensation can be seen from the data obtained for yellow and green.

The crest time of the excitability curve for green was 2 seconds at 3°, 4° and 5° from the origin, and at these points the green patch appeared colorless (Fig. 2 Green). The crest time was 1.5 seconds at 6° and 7°, and at these points the patch appeared yellow. When tested at further peripheries, the crest time was found to be 2 seconds again and in these regions the patch appeared greenish.

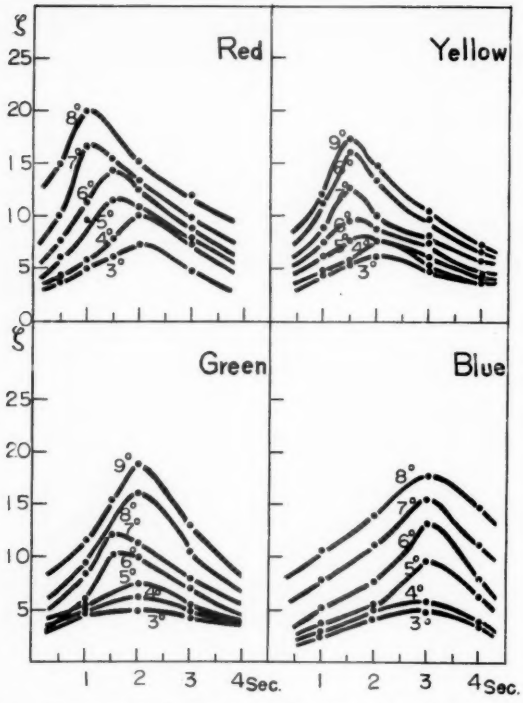


Fig. 2. The same as in Fig. 1 on peripheral side of blind spot.

However, no change occurred in the excitability curves for blue; the crest time of curves was always 3 seconds at all points tested around the spot (Figs. 1 and 2 Blue).

The blue patch appeared blue everywhere from 3° to 8°, and thus the correlation between excitability curves and sensations was as complete as in the other cases.

As is apparent from the results mentioned above, the crest time characteristic of the color used is 1 sec. for red, 1.5 sec. for yellow, 2 sec. for green and 3 sec. for blue. Conducting all points at which the crest time of the excitability curve attains a steady final form characteristic of the color used, an outer limiting line of the color-blind zone around the blind spot may be mapped.

The color-blind zones for red, green, yellow and blue lights thus constructed are illustrated in Fig. 3. The zones thus obtained are in form very similar to those determined subjectively,[5] but a little narrower than the latter. The color-blind zones for red and green are generally greater

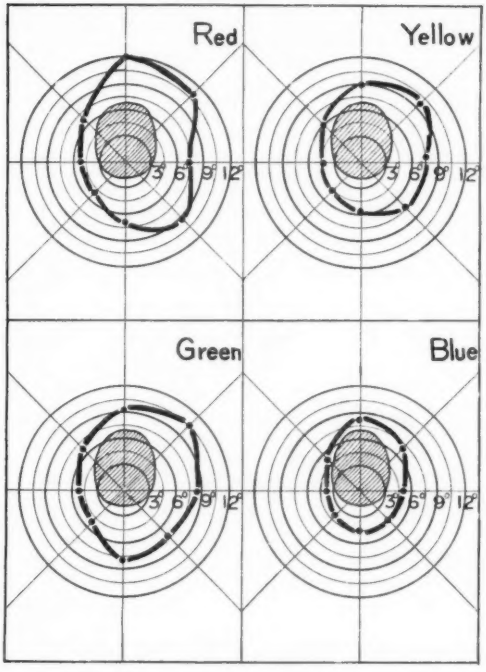


Fig. 3. Color-blind zones determined with method of electrostimulation. Further explanation in text.

than those for yellow and blue.

2. *Spatial distribution of ζ-values on either sides of the blind spot*

As can be seen from Figs. 1 and 2, ζ-values are lower near the blind spot than in remote regions.

In order to see the distribution of ζ-values near the blind spot, they are plotted against the distance from the blind spot in Figs. 4 and 5. In these figures continuous curves refer to the cone-processes, and broken ones to the rod-process. For red, yellow green and blue lights ζ-values at 1, 1.5, 2 and 3 seconds respectively were chosen to construct the continuous curves, because they represent the maximal value of each color process.

For the reason stated above, the values at 4.5 seconds were regarded as representing the rod-process, and used for construction of the broken curves.

The distribution on the horizontal meridian is shown in Fig. 4, and that on the vertical meridian (through the origin) in Fig. 5. On each

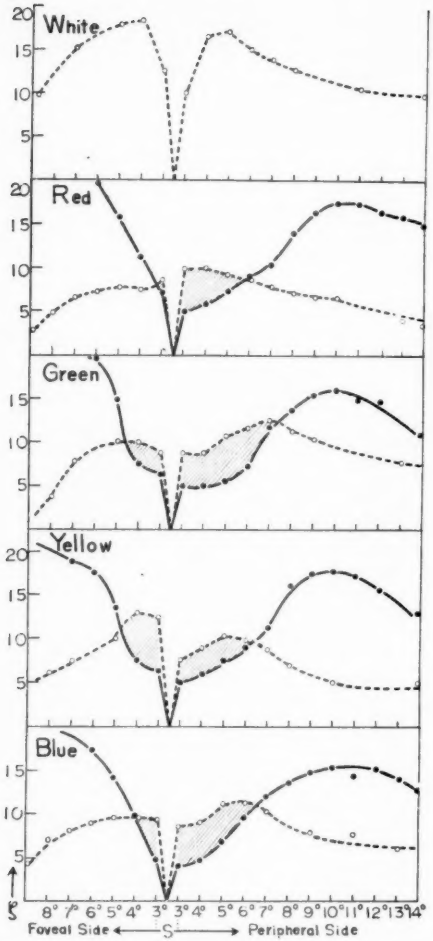


Fig. 4. Spatial distribution on horizontal meridian of cone-process (continuous curve) and of rod-process (broken curve). Ordinates: ζ-values at 1, 1.5, 2, and 3 seconds from end of illumination for red, yellow, green and blue processes respectively and at 4.5 sec. from onset of illumination for rod-process.

meridian the distributions of the cone- and the rod-processes are greatly different from each other. Generally speaking, within the color-blind zone determined above the rod-process is greater than the cone-process (see the shaded areas in Figs. 4 and 5). It may be supposed that in these areas the rod-process subserving colorless sensations is predominant over the

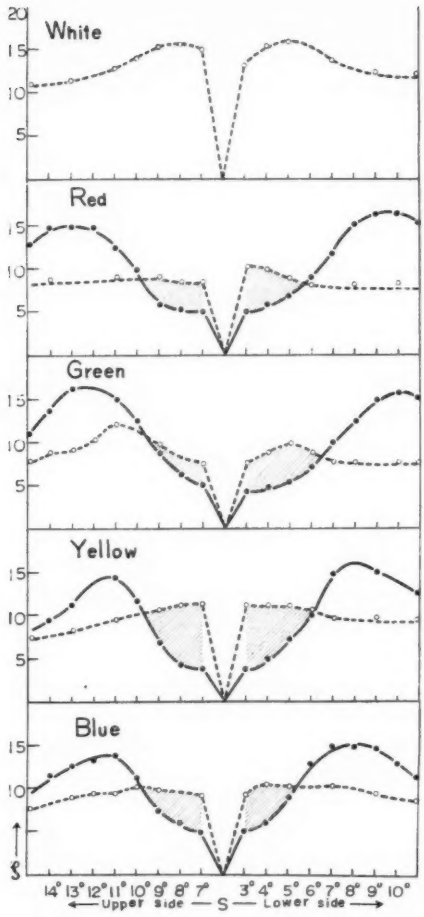


Fig. 5. The same as in Fig. 4 on vertical meridian (through the origin).

cone-processes.

Discussion

The most important thing of the results obtained above is that the color sensation at each point around the blind spot may be predicted from the form of the excitability curve obtained.

The form of the curve is not determined by the wave-length of the test light used. Near the margin of the blind spot an excitability curve having

a maximum at 2 sec. is obtained regardless of the kinds of colored light used, and this is a correlate for a colorless sensation evoked by the colored lights in this region.

If a curve having a maximum at 1.5 seconds is obtained, one may say that the patch appeared yellowish.

Moving farther away the blind spot, the curve increases in height and its crest time approaches a steady value characteristic of the color of the test light used.

The area within which the crest time is changing was called the color-blind zone. It was pointed out above that the color-blind zone determined by subjective sensations is a little wider than that determined by above-mentioned method, and this discrepancy awaits explanation.

It is likely that the excitation of rods causes desaturation of color-sensation. As is illustrated in Figs. 4 and 5, the rod-process dominates over the cone-processes around the blind spot. If this circumstance is taken into account, the discrepancy mentioned above is not difficult to understand.

Summary

The color-processes around the spot of Mariotte were investigated by Motokawa's method of electrostimulation with electrical phosphenes as index.

1. Near the blind spot colorless sensations were evoked by colored lights, and curves of electrical excitability obtained in this region were found to be independent of wave-lengths.

2. At a certain distance from the blind spot a curve characteristic of the wave-length was obtained.

The zone around the spot within which no such curve characteristic of the wave-length was obtained, was called the " color-blind zone." The extent of the zone fairly coincided with the one subjectively determined.

3. The color-blind zones for red and green lights were wider than those for yellow and blue lights.

4. Within the color-blind zone the maximum of the excitability curve shifted as the distance from the blind spot increased. But in case of blue light, no such shift occurred.

5. The distributions of cone- and rod-processes around the blind spot were investigated, and it was found that within the color-blind zone the rod-process was predominant over the cone-process.

At the end of this paper, I must respectfully express my gratitude to Prof. Motokawa for his valuable guidance in this work.

References

1) Ferree, C. E. & Rand, G., Amer. J. Ophthalmol., 1930, **13**, 690.
2) Haycraft, J. B., J. Physiol., 1910, **40**, 492.
3) Berens, C., Transact. Amer. Ophthalmol. Soc., 1923, **21**, 290.
4) Saubermann, G., Ophthalmol., 1939, **97**, 364.
5) Watanabe, K., Tohoku J. Exp. Med., 1958, **67**, 235.
6) Motokawa, K., *ibid.*, 1949, **51**, 145.
7) Motokawa, K., *ibid.*, 1949, **51**, 165, 207.
8) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
9) Motokawa, K., Tohoku J. Exp. Med., 1951, **54**, 215.
10) Oikawa, T., *ibid.*, 1953, **57**, 109.
11) Oikawa, T. & Kurosawa, T., *ibid.*, 1954, **59**, 333.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

The Characteristics of Both Hemagglutinin Inhibitors and Growth Inhibitory Substances of Influenza Viruses Found in Animal Specimens

(**Studies on several inhibitors against influenza viruses, 3rd Report**)

Nobumasa Shimizu and **Nakao Ishida**
(清水 喜正)　　(石田 名香雄)

Department of Bacteriology, School of Medicine, Tokohu University, Sendai; Director: Prof. M. Kuroya

(Received for publication, March 13, 1958)

In pursing the growth of influenza virus in animal organs, particularly by means of hemagglutinin, or in performing tissue culture growth experiment with influenza virus by means of infectivities again in tissue culture, it was thought expedient to attempt to characterize the nature of both hemagglutinin inhibitor and growth inhibitory substances detectable in animal sera or organs. How to destroy these inhibitors was also a matter of importance in performing this type of work. This paper summarizes the results obtained in experiments along these lines.

In the first series of experiments, non-specific hemagglutination inhibitors found in biological specimens were divided into four groups. The first group was the so-called β-inhibitor and was contained in bovine serum. The representative of the fourth group was guinea pig serum, and its inhibitory action was destroyed with KIO_4 but not with purified RDE. The second and the third groups were the already known α-inhibitors, but were differentiated from each other by their specific inhibitory spectrum.

In the second set of experiments, the specimens tested above were examined for their growth inhibitory activity on the PR8 and Cam virus in Maitland's type tissue cultures. Chicken chorioallantoic membrane was used as the host tissue. Except for horse serum, all sera tested contained this kind of activity against these 2 viruses. Though the elucidation of this activity was not accomplished here, further development of this type of study seems to be promising.

Finally, the third approach involved here, was to apply the results obtained above in analyzing the distribution of these inhibitors against the PR8 virus particularly in mice. The lung, kidney and spleen contained a fairly large amount of hemagglutinin inhibitors, which were susceptible to the action of RDE. Growth inhibitory substances were found only in the serum and liver of mice, and the latter was studied somewhat in detail with regard to its site of action. The inhibitory action, however, was not so high and the participation of this inhibitor in experimental infections has not been elucidated so far.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

β-Inhibitor, Its Blological and Physicochemical Properties with Particular Emphasis on the Differences from α-Inhibitor, Immune Serum and Properdin

Studies on Several Inhibitors against Influenza Viruses

2nd Report

By

Jiro Konno
(今野 二郎)

From the Department of Bacteriology, School of Medicine, Tohoku University, Sendai; Director: M. Kuroya

(Received for publication, February 3, 1958)

The first description on the non-specific hemagglutination inhibitor was made by Hirst in 1942[1], when he made the memorable discovery of the hemagglutinin with influenza viruses. The description clearly indicates that the inhibitor is rather a heat labile one.

In 1947, Francis[2] observed that if hemagglutination inhibition titration of any sera was done using heated Lee virus as antigen, an enormous titer was usually obtainable. Following up this result, Anderson *et al.*[3] reached the conclusion that a wide variety of biological materials contained substances with an inhibitory effect on influenza hemagglutinins, particularly that of the indicator state, and having a mucoid character. This inhibitor, however, was heat-stable. Since then, a good deal of work on this type of inhibitor, which is known as the Francis inhibitor, has appeared. In regards to this substance, the chemical identity with the receptor substance of red cells or the cells which would allow the virus growth was of particular interest. Not only in human serum but also in body fluids of various animals, in normal chorioallantoic fluid, in human urine and from the extract of red cells, heat-stable inhibitors have been detected and purified respectively. However, inhibitors of the Francis type do not have any neutralization activity, and this fact has been recently demonstrated by Youngner[4] using the plaque forming technic.

The other group of hemagglutination inhibitors, which are heat labile, have been detected also in the serum of various animals and its characteristics have been already described by Chu[5] and McCrea[6]. Smith[7] in 1951, proposed the term α-inhibitor to the so-called Francis inhibitor and against this, the term β-inhibitor was given to the heat

labile inhibitor. Different from the α-inhibitor, the β-inhibitor has a neutralization activity as well as inhibitory activity of hemagglutinin, but susceptible strains to this inhibitor are limited to those non-adapted to mice. With regard to the latter respect, Brans *et al.*[8] have made a detailed survey and confirmed this fact.

In 1954, Pillemer[9),10),11)] and his colleagues made a big contribution to the analysis of the so-called innate immunity. They isolated a new protein, properdin, from human serum which has a cidal action against various bacteria in the presence of complement. This substance was heat labile similary to the β-inhibitor. Ginsberg *et al.* found the virocidal activity of properdin[12)] and confirmed that the inhibitor he had previously described[13)] was identical with properdin. Carzon[14)] also isolated a hemaggulutination inhibitor against the New Castle disease virus (NDV) from human and rabbit sera and proposed the name VIS. This also was heat labile, and later concluded to be identical with properdin[15)].

In spite of the accumulation of knowledge on properdin, very little has yet emerged on the biological properties of the β-inhibitor and many people are still confused as to the difference between properdin and the β-inhibitor. This is because both of them are heat labile, are contained in animal sera and act against influenza viruses.

The work presented here chiefly concerns both the biological and chemical characteristics of the β-inhibitor of ox serum, and the difference between the α-inhibitor and properdin will be also discussed. Facts have been presented showing that the association of complement is not necessary for the action of the β-inhibitor, which clearly differentiates the β-inhibitor from properdin. Some purification studies were also performed with the former.

Experimental

Materials and Methods

Virus: The FMl strain and the CAM strain of influenza A virus were used mainly in this study. The FMl strain had been once passed through mice as can be seen in Table II and the CAM strain had been passed 30 times only in the chick embryo. The virus was prepared by inoculation of 11 day old chick embryos by the allantoic route with 0.2 m*l.* of a 10^{-5} dilution of infected allantoic fluid. The eggs were incubated at 36°C for 48 hours.

Receptor destroyind enzyme (RDE): The 4Z strain of *V. cholerae* was inoculated in peptone water containing mucin and cultivated for 16 hours at 37°C. The strain 4Z in this particular medium produced a large amount of RDE[16)] but only a small amount of β-enzyme.[17)] The obtained broth was further purified by means of adsorption to and elution from chicken red blood cells (RBC) in order to remove traces of β-enzyme.

Dry yeast and zymosan: The dry yeast used here was a commercial product. The zymosan was prepared by the method described by Pillemer *et al.*[11] 50 g. of dry yeast was suspended in one liter of 0.5 *M* Na_2HPO_4 solution and boiled for 3 hrs. After cooling, 0.2 g. of trypsin (N.B.C. 1:300) was added on the 1st, 3rd, 5th, 8th and 12th day of incubation at 37°C. To prevent contamination by bacteria, a small volume of toluene was added. After digestion with trypsin, the precipitate was collected by centrifugation. The precipitate again suspended in distilled water was heated at 100°C for 1 hour and centrifuged. This washing procedure was repeated for several times. The final precipitate was added to 1 liter of absolute ethylalcohol and dried. Without further washing in alcohol, this precipitate was used as zymosan.

Hemaggulutination inhibition titration (HAI): The two-fold dilution of serum or inhibitor were prepared in a series of 0.25 m*l.* of saline. Sufficient virus to yield 4 hemaggulutinin units in the final mixture was added to each tube in 0.25 m*l.* amounts and the mixture allowed to stand for 30 min. at room temperature. Then 0.5 m*l.* of 0.5% suspension of chicken RBC was added to each tube. The reciprocal of the final dilution of serum which inhibited hemaggulutination completely was taken as the inhibition titer.

Virus neutralization titration: To a 10 fold dilution of virus suspension, an equal volume of undiluted serum was added. After standing for 30 min. at 37°C, 0.2 m*l.* of the mixture was inoculated into fertile eggs and incubated at 35°C for 48 hours. Thus the ususal neutraliztion index (NI) was obtained by the constant serum—varying virus method.

Alternatively, the reciprocal dilution of serum which will neutralize 50% of the eggs inoculated with the 10^3EID_{50} of virus was calculated and compared with the titer of HAI. This titer was denoted as NA (neutralization activity) in the following descriptions.

Results

Limited activity of ox serum against the HA of mouse non-adapted virus

Accepting the results reported by Brans *et al.*,[8] ox serum was chosen for this study as the richest source of β-inhibitor. Different lots of pooled serum were tested for their titer of HAI against PR8, FM1, and CAM strains of type A virus and the results of a typical experiment are presented in Table I. From the results, it seems quite evident that the serum does not have any inhibitory activity against PR8 and FM1—both of them being once passed through mice in their history—but has a high titer against the mouse nonadapted CAM strain. The titer obtained with the living CAM strain was the same with that in the indicator state, which had been killed at 55°C for 30 min. These considerations would make it likely that the inhibitor detected here is not an antibody, because a quite different titer was obtained against FM1 and CAM, which are already known to have almost similar antigenic characteristics. Also the fact that the serum does not inhibit the HA of PR8 and FM1 in the indicator state suggests that the inhibitor is not the α-inhibitor.

Inhibition spectrum of the ox serum

TABLE I

Hemaggultination Inhibitors in Ox Serum

Virus	Treatment of virus	HAI titer of ox serum
PRS	alive	<32
	indicator*	<32
FMI	alive	<32
	indicator	<32
CAM	alive	1024
	indicator	1024

* Indicator virus was prepared by heating at 55°C for 30 min.

The preliminary test described above indicate that lots of ox serum obtained at different times from the slaughter house in Sendai contains the β-type but not the α-type inhibitor. In order to establish the fact, many other strains of both mouse adapted and mouse non-adapted type A viruses, type B viruses and NDV were tested. As shown in Table II, all of the mouse nonadapted strains tested, i.e. Jessup, Nakaniida, Sato and Kurafuchi were inhibited by the serum

TABLE II

Inhibition Spectrum of Ox Serum

Against influenza A viruses

Mouse non-adapted			Mouse adapted		
strains	history*	HAI titer	strains	history	HAI titer
CAM	E_{30}	1024	PRS	$F_7M_{593}E_{192}$	<32
Jessup	E_{22}	1024	FMI	$M_{37}E_{19}M_3E_{35}$	<32
Nakaniida	AE_4E_6	1024	Weiss	$F_3M_{32}E_{63}$	<32
Sato	AE_4E_1	2048			
Kurafuchi	AE_2E_4	512			
Singapore/57	AE_1E_3	<32			
Satake/57	AE_1E_2	<32			
Against influenza B viruses					
Tokyo 1-52	$AE_1E_6AE_7E_6$	<32	Lee	$F_8M_{187}E_{164}$	<32
Against New Castle disease virus					
46-9679	E_{28}	<32			

* AE and E denote amniotic and allantic inoculation in fertile eggs. M and F mean passage in mice and ferret.

at fairly high dilutions but the mouse-adapted strain, Weiss was not inhibited,

as well as PR8 and FM1. No activity was detected against influenza B and New Castle Disease viruses with this serum.

These results are all in good accordance with the report of Brans *et al.*[8] However, an exceptional type of A strain, Satake, was recently isolated during the summer epidemic in Sendai. Antigenic analysis of the strain by the HAI test revealed that the strain had a similar surface character to the A/Sinoapore/57 strain. All of the strains isolated in this epidemic, including the Singapore/57 were not inhibited by ox serum, even though they were never passed through mice. Further study on this particular problem will be reported elsewhere by Ishida *et al.*[18]

Neutralization and HAI activity

As the identity between the HAI and neutralization activity of the β-inhibitor does not seem to be fully understood, particular attention was focused to this problem throughout the study. First of all, neutralization tests were performed against PR8, FM1 and CAM strains. The results are illustrated in Table III, and parallel results with that of the HAI test have been obtained. The serum did not have any neutralization activity against two mouse-adapted

TABLE III

Neutralization Index of β-inhibitor

Virus	β-inhibitor	Infectivity/m*l.**	Neutralization index (NI) calculated	HAI activity†
PRS	Control	10.1	—	—
	With untreated inhibitor	9.9	1.5	<32
FM1	Control	9.7	—	—
	With untreated inhibitor	9.8	0	<32
CAM	Control	8.4	—	—
	With untreated inhibitor	5.9	300	1024
	With inhibitor heated at 56°C. 30 min.	7.4	10	256
	With inhibitor heated at 56°C. 1 hour	8.2	1.5	64

* The infectivity titers are expressed as the negative log of the 50% infectious dose for eggs per 1 m*l.* volume. † Assayed with 4 HAU of the CAM strain.

strains, PR8 and FM1, but had a definite titer against the CAM strain. Further studies on this problem have been performed on the heat stability of both activities against the CAM strain. The neutralization index of 300 was obtained with untreated serum (lot 2) which had the HAI titer of 1024. After heating at 56°C for 30 min. and 1 hour the decrease of both neutralization index and HAI has been followed, though the degradation constant was not fully parallel (see Table III). The dissociation of these two activities seem to require further analysis, because the neutralization test was performed by the constant serum-varying virus method.

The ratio between HAI and NA of both β-inhibitor and specific antiviral antibody.

In order to determine the difference between the β-inhibitor, and specific antibody a checker board titration was conducted as is shown in Fig. 1. As the specific antibody for comparison, rabbit immune serum against the RR8 virus was used.

Purified β-inhibitor (CAH) The activity against EID_{50} of — Rabbit immune serum (PR8) The activity against EID_{50} of

Dilution of β-inhibitor	1.0	2.0	3.0	4.0	5.0	6.0
1	○	○	○	◔	●	●
4	○	○	◕	●	●	●
16	○	◕	◕	●	●	●
64	◕	●	●	●	●	●
256	●	●	●	●	●	●

Dilution of serum	2.0	3.0	4.0	5.0	6.0	7.0	8.0
10	○	○	○	○	○	○	○
40	○	○	○	○	○	○	●
160	○	○	○	○	○	●	●
640	○	○	○	○	◕	●	●
2560	○	◔	●	●	●	●	●

The ratio between HAI and NA calculated with both β-inhibitor and antiviral immune serum.

	HAI titer	Neutralization activity (NA) against $10^3 EID_{50}$ of virus	The ratio HAI/NA
β-inhibitor	25000	4	6250
antibody	6400	6400	1

Fig. 1. Neutralization activity of both β-inhibitor and immune serum.
* In each test, 4 eggs were used. The black circle means all eggs are infected, while the white part indicates non-infected i.e. neutralized eggs.

In spite of the high HAI titer (1:25,000) of β-inhibitor precipitated by 1/3 sat. $(NH_4)_2SO_4$, the neutralization activity of it was extremely low as can be seen in the figure. If the reciprocal of the dilution of purified β-inhibitor which inhibits the growth of virus in 50% of eggs inoculated with $10^3 EID_{50}$ of virus was taken as the NA titer, only the titer of 4 was obtained with purified β-inhibitor. On the other hand the anti-PR8 immune serum which had been tested in the same way showed the NA titer of 6,400 and the HAI titer as high as 6,400. Thus if we take the ratio of the two activities, i.e. HAI as the numerator, and NA as the denominator, the ratio 1 was obtained with immune serum but a quite different ratio of 6,250 was obtained with the β-inhibitor. Though the significance of this finding cannot be interpreted at the moment, the difference itself seems to be a good tool in order to distinguish the β-inhibitor from the antiviral antibody.

The titer of β-inhibitor with different lots of ox serum

During the course of this study, many lots of serum were obtained from the slaughter house. In the fresh state, they are tested with a definite unit of HA for HAI activity and the results are summerized in Table IV. Though the deviation between individual sera has not been checked, the deviation between lot to lot is fairly small. The geometric mean value calculated from the Table is 10.3 ($-\log_2$HAI). In the Table, the HA detected with low dilutions is non-specific, and during the purification procedure it was easy to separate this spontaneous agglutinin from the β-inhibitor.

Physicochemical properties of β-inhibitor

TABLE IV

The Titers of β-Inhibitor with Different Lots of Ox Serum

Lot No.	HAI titer*											$-\log_2$ HAI
	$_2$3	4	5	6	7	8	9	10	11	12	13	
1	++†	±	−	−	−	−	−	−	−	±	++	11.3
2	++	+	±	−	−	−	−	−	+	++	++	10.0
3	++	±	−	−	−	−	−	−	++	++	++	10.5
4	+	±	−	−	−	−	−	−	++	++	++	9.3
5	++	++	++	±	−	−	−	−	−	+	++	11.0
6	++	++	+	−	−	−	−	−	++	++	++	10.5
7	+	±	−	−	−	−	−	−	±	++	++	10.3
8	++	++	±	−	−	−	−	−	±	++	++	10.3
9	++	++	++	±	−	−	−	−	++	++	++	10.5
10	++	++	++	+	−	−	−	−	++	++	++	9.0
11	++	++	+	±	−	−	−	−	++	++	++	10.5
12	++	++	+	−	−	−	−	−	+	++	++	10.0
13	++	++	++	+	±	−	−	−	−	+	++	11.0
Geom. mean												10.3

* Each lot was tested with 4 HAU of CAM strain. † indicates positive agglutination.

In order to establish the characteristics of the β-inhibitor, further study on the physicochemical properties was done with antiviral immune serum and the α-inhibitor as the control. As the immune serum, rabbit serum immunized against the CAM strain was used. As the source of α-inhibitor, undiluted horse serum was used, because it too is an animal serum analogic to the source of the β-inhibitor, and has almost the same HAI titer against the CAM strain.

1) Heat stability (see Fig. 2)

Each serum was heated in a water bath at varyous temperatures (56°, 62°, and 67°C) for 1 hour and then the residual activity to inhibit viral HA was tested. The activity of the β-inhibitor was much reduced at 56°C and was rapidly destroyed by increasing the temperature. On the contrary, the activity of the α-inhibitor was rather increased by heating, and that of immune serum

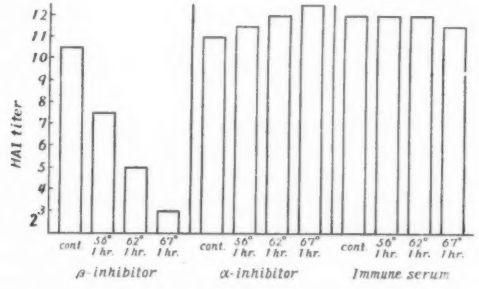


Fig. 2. Heat stability of β-inhibitor.

was not affected significantly. Thus the heat lability of the β-inhibitor seems to be an evident character, though the preservation at 4°C for as long as one month did not cause any drop of activity. The latter result seems to be somewhat different from that of Tyrell[19].

2) Influence of RDE

The purified RDE preparation with a 256 μ/ml. activity did not have any influence on the activity of the β-inhibitor similary as to that of antiviral serum when tested after incubation of 1 hour at 37°C. However, the activity of this preparation was clearly demonstrated with the α-inhibitor of horse serum.

3) Action of periodate on β-inhibitor (see Fig. 3)

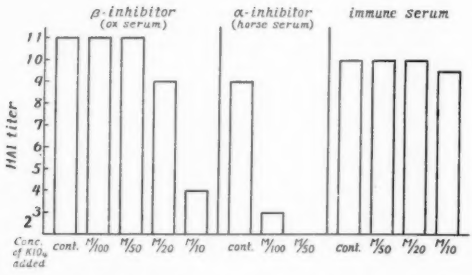


Fig. 3. Action of periodate on α-, β-inhibitors and immune serum. Each inhibitor was treated with KIO_4 at 37°C for 1 hour.

Four different dilutions of potassium periodate (0.01, 0.02, 0.05 and 0.1 *M*) were added to 3 kinds of serum in an equal volume, and after incubation at 37°C for 1 hour the mixtures were tested for their HAI activity. Before the test, residual periodate was inactivated by the addition of the same volume of 20% glucose. The activity of the β-inhibitor and antiviral serum did not change by the addition of as high as 0.05 *M* of KIO_4, while the activity of the α-inhibitor was destroyed completely by the addition of 0.02 *M* of KIO_4.

4) Action of trypsin on the inhibitors

The preparation of N.B.C., 1:300, was dissolved in the concentration of 0.25%, 0.5% and 1.0% in phosphate buffer of pH 8.0. An equal volume of these trypsin solutions were added to each inhibitor and the mixtures kept at 37°C for 1 hour. The β-inhibitor was destroyed with the increasing concentration of trypsin as well as the α-inhibitor, as can be seen in Fig. 4. The antibody was not affected by these concentrations.

5) The influence of divalent iones on the activity of β-inhibitor

Fig. 5 shows the influence of divalent iones in the test medium on the activity of the β-inhibitor. The activity of the β-inhibitor was completely abolished when examined in a 1% sodium citrate solution but the addition of 1% $CaCl_2$ to this medium did not affect the activity. Partial restoration was observed with 1% $MgCl_2$. It was evident from the result that the β-inhibitor required Ca for its action.

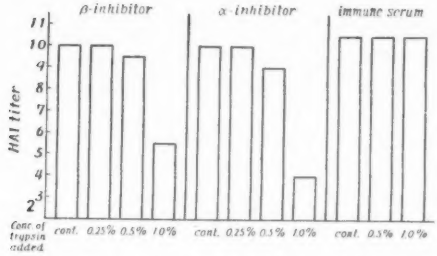


Fig. 4. Action of trypsin on α-, β-inhibitors and immune serum. Each inhibitor was treated with trypsin solution buffered at 8.0, at 37°C for 1 hour.

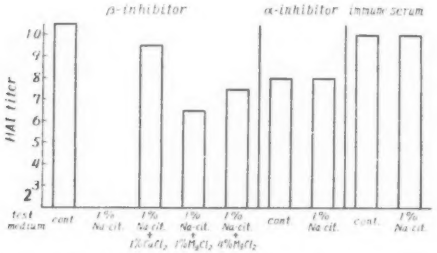


Fig. 5. The influence of divalent iones in the test medium on the activity of β-inhibitor.

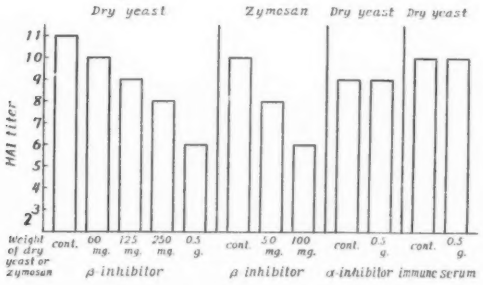


Fig. 6. Adsorption of β-inhibitor to dry yeast or zymosan. To 5 ml. of each inhibitor, dry yeast or zymosan was added and kept at room temperature for 30 min.

6) Adsorption of β-inhibitor to dry yeast and zymosan

Dry yeast or zymosan was added in amounts of 50–500 mg. to 5 m*l*. of each inhibitor, kept at room temperature for 30 min. and then centrifuged. By the addition of dry yeast the pH of the medium was reduced to 6.0, while zymosan caused no change of pH.

Treatment of the β-inhibitor with dry yeast and zymosan resulted in a reduction of activity. Later, it was found that the lowering of the titer of the supernatant was caused by adsorption, because the activity could be again extracted from zymosan with phosphate buffer of pH 7.4 (u=0.65). Thus the β-inhibitor can be purified by means of this adsorption to and elution from zymosan. Treatment of the α-inhibitor and viral antibody with dry yeast had no effect at all.

Zone electrophoresis with both α- and β-inhibitors

Electrophoretic studies were performed with α- and β-inhibitors by a method described by Kunkel and Slater[20], one for the purification and the other for the identification of each inhibitor. A starch slab 40 cm. long, 7 cm. wide, 1.5 cm. thick was made. The buffer used was barbital, pH 8.6, ionic strength of 0.05. The apparatus was kept in a cold room at 4°C. The voltage was maintained at 200 or 300 volts for 20 hours. After development, a paper strip was made by printing from the starch slab and stained with an amidoblack 10 B solution. The optical density was measured with this strip by the densitometer. Sections of the starch slab were cut in 1 cm. width and put into a tube containing 2 m*l*. of saline and vigorously shaken. 30 min. later, the clear supernatant was pipetted off and was used for HAI.

The result obtained with both normal horse serum and normal ox serum are shown in Fig. 7 and 8. The α-inhibitor activity was found in the α- and β-globuline fraction. It was clear that the β-inhibitor had a different electrophoretic mobility from that of the α-inhibitor, since the former was found between the β- and γ-globuline fraction while the latter between β- and α-globulines. Recovery rate of the β-inhibitor was usually around 30%.

β-inhibitor titers in the serum of various animals

Table V shows some efforts to utilize the result obtained above, in order to classify several inhibitors detectable in the sera of various animals. Inhibitor which are destroyed by heating at 56°C for 1 hour but do not show an alteration of titer after treatment with RDE and 1/50 *M* KIO_4 were defined as the β-inhibitor. Difference of the HAI activity against CAM and FM1, was also used for evaluation. The results seem to reveal that the sera of the ox, rabbit and mouse have the β-inhibitor and among them the ox serum shows the highest titer. The sera of the horse, guinea pig, chicken and rat have activity of the α-type but there are no traces of the β-inhibitor. The inhibitor found in the sera of the ox, rabbit and mouse, of course, has an activity against the alive CAM virus.

The purification of β-inhibitor

There has been very little work on the purification of the β-inhibitor, seemingly because of its heat lability. Study on this problem is still under way but the preliminary results will be described here. The β-inhibitor can be purified by means of salting out and adsorption to and elution from zymosan (Table VI). First, the β-inhibitor in ox serum was precipitated completely by addition of one-half the volume of saturated $(NH_4)_2SO_4$. When the precipitate was dialyzed against distilled water, the activity of the β-inhibitor was found again in the pre-

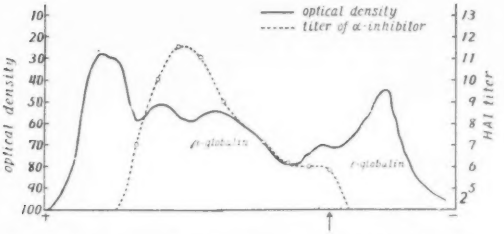


Fig. 7. The pattern of zone electrophresis with α-inhibitor in horse serum. The buffrr used was barbital, pH 8.6, ionic strength of 0.05. The voltage was maintained 200 V. for 20 hours. The arrow indicates the origin.

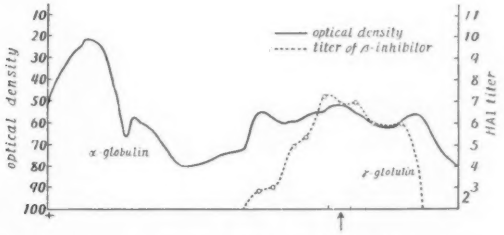


Fig. 8. The pattern of zone electrophoresis with β-inhibitor in ox serum. The buffer used was barbital, pH 8.6, ionic strength of 0.05. The voltage was maintained 300 V. for 20 hours. The arrow indicates the origin.

cipitate produced in the course of dialysis, i.e. euglobulin fraction. The recovery rate up to this stage was almost 90%. The β-inhibitor of this stage was purified about 11 times when tested by HAI titer per mg. of nitrogen and 10 times when tested by the neutralization index. In the next step, the β-inhibitor was dissolved in saline and adsorbed to zymosan at room temperature for 30 min. followed by extraction with phosphate buffer of pH 7.4 (u=0.65) at 37°C for 30 min. In this form, the purified inhibitor was more heat labile when compared to the untreated serum and decomposed even at 4°C. The activity of the eluate from zymosan was 26,300 u/mg. of nitrogen. Thus the 240 times purification was performed in the sense of activity per mg. of nitrogen. However, the analysis by the ultracentrifuge still revealed two peaks and further identification was not performed.

In another experimental run, using a separation cell of the Spinco model E, the β-inhibitor in the state of zymosan eluate, was precipitated completely in the lower cell, after centrifugation at 250,000 g. for 1 hour. Thus, it is certain that the β-inhibitor has a fairly large molecular weight.

Discussion

The HAI activity of the β-inhibitor was limited only against the mouse

TABLE V

Existence of β-Inhibitor in the Serum from Various Animals

Species	Virus	Treatment of virus	HAI taiter of			Result
			untreated serum	treated with RDE*	treated with M/50. KIO_4	
Ox	FM1	Alive	<32	—	—	Contains only β-inh.
		Indicator†	<32	—	—	
	CAM	Alive	1024	1024	1024	
		Indicator	1024	1024	1024	
Horse	FM1	Alive	64	<32	<32	α
		Indicator	1024	<32	<32	
	CAM	Alive	<32	—	—	
		Indicator	256	<32	<32	
Guinea pig	FM1	Alive	<32	—	—	α
		Indicator	512	64	<32	
	CAM	Alive	<32	—	—	
		Indicator	256	32	<32	
Rabbit	FM1	Alive	64	<32	<32	Contains β-inh.
		Indicator	2048	<32	<32	
	CAM	Alive	64	64	32	
		Indicator	1024	64	32	
Mouse	FM1	Alive	<32	—	—	Contains β-inh.
		Indicator	<32	—	—	
	CAM	Alive	32	32	16	
		Indicator	32	32	32	
Chicken	FM1	Alive	<32	—	—	α
		Indicator	128	<32	<32	
	CAM	Alive	<32	—	—	
		Indicator	64	<32	<32	
Rat	FM1	Alive	<32	—	—	α
		Indicator	512	32	<32	
	CAM	Alive	<32	—	—	
		Indicator	128	<32	<32	

* The purified RDE with 256 u/m*l* activity was used. † indicator virus was prepared by heating at 55°C for 30 min.

non-adapted strain of type A influenza virus. In addition to this fact, the neutralization activity was accompanied with HAI activity. These biological characteristics may show the difference of this inhibitor from that of the Francis type. Further, comparative studies on the physicochemical properties demonstrated heat lability and complete resistance to both RDE and KIO_4 action by the β-inhibitor.

With regard to the difference from antibody, the HAI/NA ratio was available. A titer as high as 6,250 was obtained with the β-inhibitor, while a titer of 1 was obtained with antibody. Further, trypsin treatment of β-inhibitor considerably destroyed the activity, but the same concentration of trypsin had no effect on the viral antibody at all. These biological and chemical examination are sufficient to distinguish these two substances.

Finally the discussion will be described on the difference between the

TABLE VI

Purification Procedure of β-Inhibitor

	HAI/mg. of N.
Bovine serum	110
↓ Added one-half the volume of saturated $(NH_4)_2SO_4$	
Precipitate	
↓ washed 3 times with 1/3 saturated $(NH_4)_2SO_4$	
Precipitate	
↓	
Dialysis against distilled water	
↓	
Precipitate	
↓	
Dissolve in saline	1250
↓	
Adsorbed with zymosan at room temperature for 30 minutes	
↓	
Residue	
↓	
Washed 3 times with saline	
↓	
Extracted with M/4 phosphate buffer ($\mu = 0.65$) of pH 7.4 at 37°C 30 min.	
↓	26300
Dialysed against saline	

β-inhibitor and properdin. Both are by chemical nature a protein and have a large molecular weight compared to the serum globuline. Moreover zymosan is a good adsorbent for β-inhibitor, as similary as for properdin. However, a big difference has been found with regard to the necessity of complement for the action of properdin. Though the study on properdin was not performed in this paper, numerous references[9),10),11),12)] indicate clearly the necessity of complement for the activity of properdin. However, the source of the β-inhibitor, ox serum, does not contain any hemolytic complement[21)] but still it works as an inhibitor. Further on the purification procedure, the dialysis against water or the precipitation with ammonium sulfate, which are already known to cause impairment of full complement activity, did not alter the HAI titer of the β-inhibitor. The difference existing between these 2 substances has been illustrated in Table VII.

As far as the purification study is concerned, the more purified the substance is, the easier it looses its titer. A separate study is going on at present in order to avoid this kind of degradation by addition of other substances, such as glycerol, and the possibility of the final purification depends upon the result of this study.

Summary

Biological and physicochemical properties of the β-inhibitor was studied in order to differentiate this inhibitor from the α-inhibitor, antiviral immune serum and properdin.

The substance is fairly heat-labile and susceptible to trypsin action, indicating the protein nature. However these characteristics are quite

TABLE

Differentiation of

	Necessity of complement in action	Activity when dialysed against water	Activity when precipitated with $(NH_4)_2SO_4$	Temperature of reaction	Optimum pH range of reaction
Properdin	Yes	No activity	No activity	Reaction proceeds only above 15°C	6.5–8.2
β-inhibitor	No	Full activity	Full activity	Reaction proceeds even at 4°C considerably	6.0–8.0

different from that of specific immune serum. The β-inhibitor is resistant to the action of both RDE and KIO_4, thus distinguishing it from the α-inhibitor. Though the adsorption to zymosan has been proved with this agent as has been already proved with properdin, the participation of complement for the action of the β-inhibitor is not necessary at all. Further, biological activity of the β-inhibitor is limited just to the mouse non-adapted type A influenza viruses.

The richest source of β-inhibiror was ox serum, but it was also detected in the serum of the rabbit and mouse. The purification of the β-inhibitor was performed from ox serum and after precipitation with ammonium sulfate, adsorption to and elution from zymosan was particularly effective to remove the other entities contained in ox serum. Though the 240 times purification was achieved in the sense of HAI activity per mg. of nitrogen, the heat lability increased when the purification advanced. From this reason, the purification study is still going on.

ACKNOWLEDGMENTS

The author wishes to thank Dr. M. Kuroya and Dr. N. Ishida for their helpful advice and stimulating discussions.

References

1) Hirst, G. K., J. Exp. Med., 1942, **75**, 49.
2) Francis, T. Jr., *ibid.*, 1947, **85**, 1.
3) Anderson, S. G., Burnet, F. M., Fazekas, de St. Groth, S., McCrea, J. F. & Stone J. D., Australian J. Exper. Biol. & M. Sc., 1948, **26**, 403.
4) Henry, C. & Youngner, J. S., J. Immunol., 1957, **78**, 273.
5) Chu, C. M., J. Gen. Microbiol., 1951, **5**, 739.
6) McCrea, J. F. Australian J. Exper. Biol. & Med. Sc., 1946, **24**, 283.
7) Smith, W., Westwood, J. C. N. & Belyavin, G. Lancet, 1951, **2**, 1189.
8) Brans, L. M., Hertzberger, E. & Binkhorst, J. L., Antonie van Leeuwenhoek, 1953,

VII

β-Inhibitor from Properdin

Requirement of divalent ion	Heat stability of untreated serum	Heat stability of purified preparation	Activity of serum from various animals	Inhibition spectrum
Mg^{++}, No description on Ca^{++}	Destroyed completely at 56°C after 30 min.	Becomes more stable, stable at 66°C after 30 min.	Rat 25–50 u/m/. Mouse 10–20 Cow 10–20 Rabbit 4– 8	Has a wide spectrum including inf. A, B and NDV
Requires Ca^{++} rather than Mg^{++}	Destroyed at 62°C almost after 1 hr.	Becomes more labile, completely destroyed at 56°C after 30 min.	Ox 1024HAI/m/. Rabbit 64 Mouse 32 Rat 8	Limited to only against mouse-non-adapted strain of type A influenza virus

19, 309.

9) Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W. & Wardlaw, A. C., Science, 1954, **120**, 279.

10) Pillemer, L., Schoenberg, M. D., Blum, L. & Wurz, L., *ibid.*, 1955, **122**, 545.

11) Pillemer, L., Blum, L., Lepow, I. H. & Wurz, L., J. Exp. Med., 1956, **103**, 1.

12) Ginsberg, H. S., & Wedgwood, R. J., Annals of the New York Academy of Sciences, 1956, **66**, 251.

13) Ginsberg, H. S. & Horsfall, F. L. Jr., J. Exp. Med., 1949, **90**, 475.

14) Carzon, D. T., J. Immunol., 1956, **76**, 454.

15) Carzon, D. T., Personal communication to Dr. Ishida.

16) Kawamura, K., Virus, 1958, **8**, 59.

17) Kawamura, K., unpublished results.

18) Ishida, N. et al., Sogo-Igaku, 1958, **15**, 51.

19) Tyrrell, D. A. J., J. Immunol., 1954, **72**, 494.

20) Kunkel, H. G. & Slater, R. J., Proc. Soc. Exper. Biol. & Med., 1952, **80**, 42.

21) Bier, O., J. Immunol., 1945, **51**, 147.

The Tohoku Journal of Experimental Medicine, Vol. 67, No. 4, 1958

Optimum Conditions for the Action of Receptor Destroying Enzyme on Red Cells and Ovomucin

(Studies on several inhibitors against influenza virus, 4th Report)

Keiji Kawamura
(川村 慶二)

The Department of Bacteriology, School of Medicine, Tohoku University, Sendai; Director: Prof. M. Kuroya

(Received for publication, March 13, 1958)

In a previous paper[1], some purification studies on the receptor destroying enzyme (RDE) produced in a particular medium containing hog stomach mucin, which allowed the highest production of RDE by V. cholerae, have been reported.

For the application of this purified preparation on serological studies of influenza infections and also for detecting the viral hemagglutinins in the organ of animals infected with influenza virus, systemic research on the optimum conditions for the action of RDE seemed advisable. The results of basic studies obtained along this line are the subject of this communication.

1) Using chicken red cells and ovomucin as the substrates, quantitative studies of RDE activity have been performed. The higher the concentration of the substrate, the higher concentration of enzyme was necessary. Linear relationship was obtained between the log concentration of the substrate and of enzyme in order to destroy the activity of the substrate completely after a fixed period of incubation.

2) Optimum temperature and pH for the action of RDE was 37°C and between 6.5 and 7.0, respectively.

3) Ca ion concentration was an important factor for the RDE action, with the optimum concentrations situated between M/100 and M/200. Deionization with EDTA, sod. citrate and sod. oxalate inhibited the RDE action and the effectiveness of these substances was in the same order.

4) The provisional working standard for determining the titer of both α-inhibitor and RDE has been proposed. Following this standard, x/4 unit of RDE was necessary to destroy the x unit of α-inhibitor under standard conditions of incubation, i.e. at 37°C for 1 hr.

5) Methods to remove the residual activity of RDE after the completion of the reaction have been examined. Heating was favourable for practical use, particularly at the time of serodiagnosis. However, there was a difference of heat stability between purified RDE and simple cholerae filtrate. The assay with 2.5% sod. citrate saline, which has been customly used as the routine procedure to remove the residual RDE activity, was not so satisfactory when a high concentration of RDE was used. A 1% aqueous solution of EDTA was preferrable with regard to this respect.

The conditions determined above gives an idea of how many units of RDE should be used in order to remove a known amount of α-inhibitor contained in the specimens to be tested, and also how to remove the residual activity of the used RDE. However, the existence of other kinds of α-inhibitors among the biological specimens which are not impaired by the above procedure, was not ruled out[2].

References: 1) K. Kawamura, Tohoku J. Exp. M.; 1958, **67**, 372.. 2) N. Shimizu & N. Ishida, *ibid.*, 1958, **67**, 390.